# RADIOIMMUNOLOGICAL AND CLINICAL STUDIES WITH LUTEINIZING HORMONE RELEASING HORMONE (LRH)

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PROEFSCHRIFT
ter verkrijging van de graad van
doctor in de wiskunde en natuurwetenschappen
aan de Katholieke Universiteit te Nijmegen,
op gezag van de Rector Magnificus Prof.Dr. J.H.G.I Giesbers
volgens besluit van het College van Decanen
in het openbaar te verdedigen op woensdag
26 november 1986 des namiddags te 1.30 uur precies

door

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geboren te Karlstad, Zweden

Delta Drukkerijen.
Zutphen 1986

SERO SED SERIO

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This thesis was initiated quite some time ago and over the years a considerable number of people were involved in the long process of it becoming reality. The rules of the University of Nijmegen does not permit me to thank the person who in fact made the whole project possible. Rather then making a long list of names, that would be incomplete, I would like to say: thank you all, no one named but absolutely none forgotten. Some of your contributions were absolutely indespensible, some were utterly necessary, some were "merely" very useful. Certain is that that without all of you it would never have been possible.

# CHAPTER 1

## Introduction

### 1.1 Aims and objectives

This study was undertaken in order to establish a detection system for luteinizing hormone releasing hormone (LRH) to facilitate in vivo and in vitro studies with this compound. Furthermore it was the intention to investigate conditions for testing the pituitary response to hypothalamic stimulation in man.

Regulation of the reproductive function has been extensively studied on the gonadal level and to a lesser extent on the pituitary level. The structural identification of LRH as an oligopeptide (Schally et al. 1971a, Currie et al. 1971, Burgus et al. 1972) permitted the development of radioimmunoassay techniques for this hormone. It also provided the means for a test of the functional capacity of the pituitary. In turn these two factors opened up the higher centres of reproductive control mechanisms to more direct studies.

In this thesis we shall describe the development and characterization of a radioimmunoassay for LRH (chapters 3, 4 and 5) as well as measurements of the hormone in the general circulation (chapter 6), and also in tissues and in incubation media from in vitro studies (chapter 7). In chapter 8 we describe how the pituitary reacts in terms of gonadotrophin release in response to LRH administered in different ways and under various conditions.

The studies were carried out at the University of Ulm during the author's tenure as head of the Peptide Hormone Laboratories of the Department of Obstetrics & Gynaecology between 1971 and 1977. Most of the results have previously been published in original publications (Dahlen, Keller & Schneider, 1974; Dahlen & Schneider, 1975; Dahlen, Voigt & Schneider, 1975 and 1976; Schneider & Dahlen, 1972a, 1972b, 1973a, 1973b, 1973c and 1975). Our results are being discussed in this thesis in the light of subsequent publications of other investigators in this field. The application of radioimmunoassays to the study of reproductive neuroendocrinology has made during the past decade such great advances that the radioimmunoassay method has changed from a rather specialized technique used in only few laboratories to a well established and widely used routine method. In the process, this technique has revolutionized the entire discipline of endocrinology. Hence, while presenting our experiments, and the motivation for doing them, in the light of the state of knowledge in the seventies, we discuss our observations in the light of current knowledge.

## 1.2 General physiological background

### 1.2.1 Anatomy of the hypothalamo-pituitary system

The hypothalamus is a small part of the brain lying in the base and forms the floor of the 3rd ventricle and parts of its walls. It is a diffuse area without precise boundaries, but it can be divided into groups of nuclei on the basis of physiological functions. The hypothalamus joins the pituitary at a junction known as the median eminence. The pituitary stalk connects the hypothalamus to the pituitary gland. The pituitary gland is divided into two lobes, the anterior lobe or the adenohypophysis, and the posterior lobe or the neurohypophysis. In some species there is also an intermediate lobe. The neurohypophysis is connected to the hypothalamus by a large number of neurons. The adenohypophysis, however, is not innervated. Communication to higher centres is provided by a set of blood vessels, referred to as the hypothalamo-hypophysial portal vessels. These vessels are in close contact with terminals of hypothalamic nerve fibres in the median eminence and they distribute blood throughout the anterior pituitary.

### 1.2.2 Physiological role of the hypothalamus

The hypothalamus is a small but important region of the brain. It is concerned with a number of vital functions such as water metabolism, temperature regulation, pituitary secretion and it thus controls the gonads and the thyroid and adrenal glands. It also controls the secretion of growth hormone and prolactin.

By tradition, hypothalamic action is divided into two parts on the basis of functional criteria. The first action is localized in the supraoptical and paraventricular nuclei and is concerned with the control of water metabolism, milk ejection during lactation and uterine contractions at partus. This function is performed by means of neurosecretory cells in the above mentioned nuclei. These neurons project down into the posterior pituitary and are capable of secreting the hormones which are responsible for the control of the above mentioned events. The hormones are produced in the hypothalamus and pass down the axons to the nerve terminals in the posterior pituitary where they are released into the general circulation to reach their target tissues.

The other part of the hypothalamic action is referred to as the hypophysiotrophic function. This system consists of special neurons which produce hormones and transport them to nerve endings in the median eminence region. Here, the neurohormones enter the

blood stream in the capillary bed of a portal system. The hormones are then carried through these portal vessels into the anterior pituitary, where they cause the parenchymal cells to release or withhold their respective hormones. This vascular network is connecting the basal medial hypothalamus with the anterior pituitary, thus linking the central nervous system with the endocrine system. This is a unique type of delivery system, which requires only minute amounts of the hormone to be secreted. One consequence of this is that these hormones are greatly diluted upon reaching the general circulatory system. This makes their detection in the periphery a more than usually difficult matter.

Apart from the above mentioned two groups of hypothalamic peptides, we now recognize that several other peptides are in fact found in vertebrate hypothalamic tissues. For instance, recently the endogenous opiate peptides (endorphins and enkaphalins) as well as Substance P and neurotensin have been identified. These hormones have also a widespread, albeit uneven, extra-hypothalamic distribution and their physiological roles are as yet largely speculative. Recent studies indicate, relevant to these studies, modulatory effects of endogenous opiates on the feed-back control by gonadal steroids on LRH release from the hypothalamus (Bruni et al. 1977, Drouva et al. 1981).

### 1.2.3 Hypothalamic releasing and inhibiting hormones

Hypothalamic releasing and inhibiting hormones are defined as substances of hypothalamic origin which stimulate or inhibit the release of the anterior pituitary hormones. The existence of such a control mechanism was suggested by the early studies of Harris and collegues (Green & Harris, 1947; Harris, 1948; Harris, 1955) and has since then been extensively investigated and proven.

Since other cells of the hypothalamus were known to secrete the peptide hormones antidiuretic hormone and oxytocin, it was thought that the releasing hormones and inhibiting hormones would also be small polypeptides. Consequently, the purification of the hypothalamic extracts was carried out by means of classical polypeptide purification methods. Starting material were hypothalami collected in large quantities from local abattoirs, which were immediately frozen. The tissues were then subjected to lyophilization, delipidation, extraction with acetic acid, chromatography on ion-exchange resins, partition chromatography, electrophoresis, counter current distribution and high voltage zone electrophoresis. The isolated peptides were subjected to amino acid analysis and amino acid sequence determination (Schally et al. 1971a). In order to be able to

monitor the purification process, it was necessary to develop specific bioassays for the anterior pituitary hormones (McKenzie, 1958; Kliss et al. 1963; Steelman & Pohley, 1953; Greenspan et al. 1949; Parlow, 1961; Nicoll et al. 1970; Nicoll, 1972). Subsequently these were replaced by radioimmunoassays (Monroe et al. 1968; Niswender et al. 1968; Hendrich et al. 1971; Landon et al. 1967a). Specific assays for the releasing hormones were also developed (Ramirez & McCann, 1961; Saffran & Schally, 1955; Igarashi & McCann, 1961; Redding et al. 1966; Pecing et al. 1965).

The first releasing hormone to be chemically identified was the thyrotrophin releasing hormone (TRH). The group of Guillemin reported the isolation of 1 mg pure TRH from 300,000 sheep hypothalamic fragments (Burgus et al. 1969). Almost simultaneously Schally and his collaborators (Boehler et al. 1969) reported the purification of the porcine TRH. This hormone is composed of only three amino acids : glutamic acid, histidine and proline in equimolar ratio (Schally et al. 1969a). Further studies led to the definition of the structure and its synthesis (Folkers et al. 1969; Schally et al. 1969b). Comparisons between synthetic and the natural hormones proved that they were identical (Bowers et al. 1970). It has also been shown that this tripeptide (L-pyro-glutamyl-L-histidyl-L-proline amide) is capable of releasing thyrotrophin in all mammals studied so far. An intact amide and the cyclized glutamic acid terminal are essential for activity. The hormone will also release prolactin in man and many animals (Bowers et al. 1971; Jacobs et al. 1971).

McCann, Taleisnik · & Friedman (1960) demonstrated the existence of a luteinizing hormone releasing hormone in hypothalamic extracts. Extensive physiological and biochemical studies in several laboratories culminated in the isolation of pure LRH from porcine hypothalami by Schally et al. (1971a), from bovine hypothalami by Currie et al. (1971) and from ovine hypothalami by Burgus et al. (1972). Acid hydrolysis of the peptide yielded 9 amino acids while the alkaline and acid hydrolysis in the presence of thioglycollic acid showed the presence of a 10th amino acid, tryptophane, which is destroyed by acid hydrolysis (Schally et al. 1971b). Further studies indicated a blocked N-terminal and an aminated C-terminal, as in the case of TRH. Extensive physiological studies showed that the synthetic product with the proposed structure (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) was identical to pure, natural LRH (Kastin et al. 1972; Schneider & Dahlen, 1972a; Nillius & Wide, 1972; Dahlen, Keller & Schneider, 1974; Schally et al. 1976). The hormone was found to be able to elicit not only the release of luteinizing hormone (LH), but also that of follicle stimulating hormone (FSH) in man and many other species (Schally et al. 1976 and many others).

Scarcity of material prevented amino acid sequencing in many species, but the combined immunologic, chromatographic and physiologic evidence indicates that this decapeptide is the LH-releasing hormone of all mammals, including man. The synthetic decapeptide has been shown to release both LH and FSH in a large number of species including rat, mouse, rabbit, hamster, mink, horse, cattle, pig, chicken, pigeon, trout, carp, monkey and man (Schally et al. 1973, 1976, 1978a & b; Wade 1981). Chromatographic evidence suggests that although the mammalian decapeptide is capable of releasing gonadotrophins in these species, slight modifications in the structure may be found in the avian, reptilian, amphibian and fish releasing hormone (King & Millar, 1980). This was confirmed for chicken LRH, which differs from mammalian LRH in that the arginine in position 8 is replaced by glutamic acid (Miyamoto et al. 1982; King et al. 1982a & 1982b). A second species of chicken LRH with three amino acids substituted (histidine in position 5, tryptophane in position 7 and tyrosine in position 8) has also been identified (Miyamoto et al. 1984). Sherwood et al. (1983) found a separate teleost LRH differing in two positions (tryptophane at 7 and leucine in 8).

Growth hormone release inhibiting hormone (GIH or somatostatin) was the next hypothalamic "releasing hormone" to be identified. It was isolated by the group of Guillemin (Brazeau et al. 1973) from ovine hypothalamic extracts and by Schally et al. (1976) from the porcine hypothalamus. The primary structure (Ala-Gly-Cys-Lys-Asn-Phe-Trp-Lys-Thr-Ser-Cys with a -S-S- link between the two cysteine moieties forming a cyclic peptide) is identical in both species. Larger biologically active forms were found in the pig hypothalamus by Schally et al. (1976) and in extracts of rat pancreas, stomach and duodenum by Arimura et al. (1975). It was initially thought that these forms may represent precursors of the hormone (Schally et al. 1979), but later it could be established that the larger forms of the hormone are also secreted and are in fact biologically active (Rorstad et al. 1979, Mandarino et al. 1981).

Corticotrophin releasing hormone (CRH) was the first hypothalamic releasing hormone whose existence could be proven (Guillemin & Rosenburg, 1955). However, it took many years before the chemical identity of ovine CRH could be elucidated by Vale et al. (1981). This was followed by the structure of the murine releasing hormone by Rivier et al. (1983) and the human hormone by Shibahama et al. (1983). These releasing hormones are all peptides with 41 amino acids. The human and rat releasing hormones are identical, whereas the ovine hormone differs in 7 amino acids.

Growth hormone releasing hormone (GRH) activity was claimed by Schally et al. (1969b) for a decapeptide isolated from porcine hypothalamic fragments on the basis of controversial and complicated bioassays (tibia test and pituitary depletion test). The physiological significance of the substance is still in doubt. The amino acid sequence is similar to that of a part of haemoglobin and it could be an artifact. Recently, however, the releasing hormone could be isolated from a human pancreatic tumour from a patient with acromegaly. It was identified as a 44 amino acid polypeptide by Guillemin et al. (1983). A similar structure could be shown by Esch et al. (1983) for the bovine and by Boehler et al. (1983) for the porcine GRH. The structure of murine GRH proved to deviate considerably more from the above peptides (Spiess et al. 1983).

Various physiological and clinical observations can best be explained by the presence in the hypothalamus of a specific FSH releasing hormone (Igarashi & McCann, 1964). The question of the existence of a hormone distinct from the above mentioned decapeptide, which stimulates both the LH and FSH secretion, is still open. Some prominent researchers in the field now discard the idea of the existence of such a hormone (Schally et al. 1979), whereas e.g. McCann (1983a & 1983b) still argues in the favour of the existence of a separate FSH-releasing hormone. More and more evidence in favour of the latter conclusion are accumulating. For instance, recent studies with LRH antiserum indicate two separate system for FSH release: the decapeptide referred to above and another immunologically different entity (Culler & Negro-Vilar, 1986)

As regards the existence of a prolactin releasing hormone (PRH), it has already been mentioned that TRH is able to elicit prolactin release. It remains to be seen whether TRH is the physiological releasing hormone. There is some evidence for the existence of a different hormone. Partially purified hypothalamic fractions, which do not appear to contain TRH, can stimulate the release of prolactin from pituitaries in vivo and in vitro (Schally et al. 1973; Vale et al. 1977; Boyd et al. 1976). A compound known to have prolactin releasing hormone activity is the octosapeptide vasoactive intestinal polypeptide (VIP). This compound can cause the release of prolactin in vivo in intact animals (Kato et al. 1978) and in stalk-transected monkeys (Frawley & Neill, 1981). Similar results were obtained in vitro with pituitary tissue (Ruberg et al. 1978; Shaar et al. 1979; Frawley & Neill,1981).

A prolactin release inhibiting hormone (PIH) has been demonstrated by Talwalker, Ratner & Meites (1963) in rat hypothalami.

It was detected in other species by a number of investigators (Schally et al. 1968). Hypothalamic extracts, highly purified in terms of bioassayable PIH, have been shown to contain large amounts of catecholamines. These are known to be able to inhibit prolactin secretion (MacLeod, 1969; Takahara et al. 1974). Administration of catecholamine inhibitors leads to incresed prolactin release (Donoso et al. 1971; Friesen et al. 1972; Frohman & Stachura, 1975). L-DOPA, a precursor of dopamine, suppresses the prolactin output (Frohman & Stachura, 1975; Malarkey et al. 1971), as do the dopamine receptor antagonists apomorphine (Martin et al. 1974) and bromocriptin (Del Pozo, 1974). Injection of apomorphine into the 3rd ventricle also supresses prolactin release (Vijayan & McCann, 1978a) There is little doubt that dopamine can act directly on the pituitary to inhibit prolactin release. MacLeod & Lehmayer (1974) have shown that this biogenic amine can inhibit prolactin release by the pituitary in vitro. In stalk-transected monkeys infusion of dopamine in physiological amounts lowered prolactin levels to approximately normal values (Frawley & Neill, 1983). Dopamine has been detected in the portal vessels by Ben-Jonathan et al. (1977), and thus it is conceivable that dopamine is the prolactin release inhibiting hormone.

### 1.2.4 Biosynthesis of LRH

Neuropeptides are released by neurosecretory cells at sites located at the end of often very long axons projecting into the median eminence in the case of LRH secreting neurons. In a neuron the ribosomes and endoplasmatic reticulum are almost entirely confined to the perikarya. This implies that neuropeptides, such as LRH, must either be synthesized locally at the site of release by a non-ribosomal, mRNA-independent process or that it is synthesized in the perikarya in the conventional manner and then transported down the axons before being released. Evidence for non-ribosomal biosynthesis in prokaryotes has been found for small peptides such as gramicidine S, thyrocidine and the polymyxin-group of antibiotics (Lipman, 1971 and 1973; Laland, 1972; Kurahashi, 1974). In eukaryotes non-ribosomal synthesis has been reported for small peptides such as glutathione (Meister, 1973a & 1973b).

Initial experiments concerning the identity of the biosynthetic mechanism involved in the production of LRH pointed to a non-ribosomal type (Johanson et al. 1973). This was also reported for GRH (Reichlin & Mitnick, 1973), TRH (Mitnick & Reichlin, 1971) and PRH (Mitnick et al. 1972). However, over the years evidence accumulated for the more ubiquitous ribosomal type of synthesis. E.g. Millar et al.

(1977) reported higher molecular forms of LRH , which are more likeiv to be the product of ribosomai synthesis. Gel chromatography on Sephadex G-25 of sheep hypothalamic extracts revealed three peaks of LRH actvity, one co-migrating with synthetic LRH and two containing higher molecular weight compounds. The latter could be converted into LRH-like material by means of exposure to peptidases but not by urea, indicating a peptide-structure rather than an aggregate structure. It is, therefore, possible that the two higher molecular compounds may in fact be precursors for LRH. Such precursors have been reported for many hormones and other secretory products (Gainer et al. 1977, Loh & Gainer 1983).

Further evidence for the existence of LRH precursors was supplied by Galton, Pattou & Kordon (1981). They could show the presence of large molecular weight compounds related to LRH, not only in hypothalamic extracts, but also in cortical and placental extracts. Two forms were indicated, one with a molecular mass of about 1.8 kD (i.e. about twice that of LRH) and one with a molecular mass of approx. 26 kD. The large form was found in cytoplasmatic and axoplasmatic fractions, the smaller compound was located in the axoplasmatic and synaptosomal fractions and the decapeptide almost exclusively in the synaptosomal fractions.

The existence of a precursor for LRH was recently confirmed using modern DNA cloning and sequencing techniques (Seeburg & Adelman, 1984). A sequence in human DNA was found which coded for a 92 amino acid sequence, preceded by a signal peptide in the expected manner, containing not only the decapeptide LRH sequence but also the expected amino acid sequences for C-terminal amidation and processing to LRH. The evidence, from these and the earlier experiments, suggests that LRH is synthesized as one or more large precursors in the perikarya, is transported down the axons and appears in the nerve terminals as the decapeptide, probably as the result of enzymatic processing.

In analogy to the posterior pituitary peptides (Browstein et al. 1980), the releasing hormone may be moved to the nerve terminals in granules that are being transported down the axons of the LRH secreting neurons. LRH containing granules have been demonstrated both in perikarya and in nerve endings (Goldsmith & Ganong, 1975; Silverman & Desnoyers, 1976; Kozlowski et al. 1980). Also favouring axonal transport is the effect of colchicine, which is known to block this type of mechanism (Schwartz, 1980). Addition of this drug in vitro decreases the release of LRH from hypothalamic fragments into the medium (Hartter & Ramirez, 1980).

The release of LRH from the nerve terminals can be activated by depolarization or by direct electrical stimulation or by elevated

potassium levels The release depends on the external calcium concentration and requires energy (Hartter & Ramirez, 1980, Drouva et al 1981, Dyer et al, 1980)

1.2 5 Control of releasing hormone secretion

One aspect of hypothalamic control of hypophyseal activities is the integration of a complex system of hormonal and neural inputs into coordinated outputs In the case of the control of gonadotrophin secretion there are external inputs such as photoperiod (in seasonal breeders or circadian rhythms), olfactory inputs (pheromones), tactile inputs (copulation-induced ovulation) In addition there are internal inputs from other hormones, e g effects of prolactin and steroid hormone or gonadotrophin feed back All these inputs must be integrated and compiled into one or more signals, which will lead to the required gonadotrophin secretion pattern This integration is thought to be achieved by a complex network of neural connections inside and outside the hypothalamus

The role of neurotransmitters is essentially indirect, but it is also true that almost any neuroendocrine function can be affected by manipulation of neurotransmitters The main chemical neurotranmitters - dopamine, norepinephrine, epinephrine, serotonin and acetylcholine - are all assumed to modulate interneural transmission. These mediators are thought to be involved in transfering signals from other brain centres to the hypothalamic hypophysiotrophic neurons.

Extrahypothalamic nerve structures may influence the peptidergic neurons in the hypothalamus (Halasz, 1969, Halasz, 1972, Sawyer, 1975), although these neurons are capable of sustaining certain autonomous functions Many, but not all, pituitary trophic hormone functions are maintained when the hypothalamus is disconnected from the rest of the brain E g basal LH levels are maintained and the normal pulsatile manner of release of the hormone from the pituitary is also observed (Blake & Sawyer, 1974, Soper & Weick, 1980). The ovulatory surge of this hormone, however, is lost (Krey, Butler & Knobil, 1975) However, when placing lesions within the arcuate nucleus gonadotrophin levels fall to very low (Plant et al 1978), comparable to post pituitary stalk section (Vaughan et al 1980) These findings suggest that though some hypothalamic functions can occur in isolation, stimulatory and/or inhibitory inputs from other areas of the brain are needed for full and complete function of the hypothalamus These studies and many others lead to the concept of a dual level control of anterior pituitary function One system regulates the basal secretion and the other regulates all other

functions such as diurnal rhythms and ovulatory gonadotropnin surge (Hutchinson, 1978).

The first evidence concerning the nature of the neurotransmitter involved in the control of gonadotrophins (via releasing hormones) was the finding that adrenergic and cholinergic blockers could prevent ovulation (Sawyer, Markee & Everett, 1950) Later investigations by Schneider & McCann, (1969 and 1970a, 1970b, 1970c) indicated that dopamine is involved This synaptic transmitter is able to stimulate the release of luteinizing hormone. At the same time, however, evidence for a decreased turnover of tuberoinfundibular dopamine was presented by Fuxe & Hoekfelt (1969) Over the years the opinion shifted from a stimulatory role of dopamine to an inhibitory one Recent findings suggest that dopamine is involved both in the stimulation as well as the inhibition of gonadotrophin release Which of the two actions prevails at a given moment probably depends on the steroid environment (McCann, 1983a) It has also been demonstrated that high doses of the amine can inhibit LH release (Vijayan & McCann, 1978a, Gnodde & Schuiling, 1976)

It appears to be established beyond reasonable doubt that norepinephrine is involved in the stimulatory effects leading to the release of LH This has been concluded from the studies of many investigators over a long period of time Donoso et al (1970), Bapna et al (1971), Chiocchio et al (1976), Selmanoff et al (1977), Vijayan & McCann (1978a), Negro-Vilar (1979), Rance et al (1981), Ojeda et al (1982). These studies were carried out by blocking neurotransmitter receptors or enzymes involved in catecholamine biosynthesis or by determining catecholamine turnover

Acetylcholine increases FSH release in vitro from incubated hypothalamo-pituitary tissue Its intraventricular injection induces LH release from the pituitary (Fiorindo et al. 1975, Vijayan & McCann, 1980). Since atropin can block this effect it seems that cholinergic neurons and muscarinic receptors play a role in the control of LH release. Furthermore, since the response to acetylcholine can be blocked by addition of a dopamine receptor blocker (Vijayan & McCann, 1980), it seems likely that a dopaminergic neuron is also involved in this effect Three other neurotransmitters have been implicated in the system: serotonin, gamma-aminobutyric acid and histamine (Schneider & McCann, 1970, Hery et al 1976, Libertun & McCann, 1976, McCann et al. 1981). The physiological significance of these findings cannot be assessed at this time

Summing up the available evidence McCann (1983b) postulates that both norepinephrine and dopamine are involved in the preovulatory LH surge initiating ovulation, and that a complex interplay of noradrenergic, cholinergic and dopaminergic neurons is involved in the

regulation of the pulsatile release pattern of LH (i.e. maintanance of basal levels).

Over the years overwhelming evidence has accumulated favouring dopaminergic inhibitory control of prolactin secretion (see above in section 1.2.3). However we must distinguish between dopamine as a neurotransmitter and as a "releasing hormone", and it seems that although this biogenic amine is undoubtably involved in the inhibition of prolactin release, the site of action is probably the pituitary and not any of the higher centres. Serotonin precursors release prolactin when administered to man (MacIndoe & Turkington, 1970) and rat (Kamberi, Schneider & McCann, 1970). Blocking of the 5-hydroxytryptophane synthesis prevents suckling-induced prolactin release in the rat (Kordon et al. 1973). Thus it is possible that stimulatory effects on prolactin release occurs via a serotonergic pathway.

It seems that adrenergic and noradrenergic pathways are involved in the control of growth hormone secretion Increases of hypothalamic GIH output have been demonstrated in vitro (Negro-Vilar, 1978) and in vivo (Vijayan, Krulich & McCann, 1975; Turkelson et al. 1979). On the other hand, adrenergic and noradrenergic stimulation has also been shown to be occurring via GRH (Eden et al. 1981; Terry & Martin 1981). It is difficult to reconcile these results. A dopaminergic pathway has been implicated by the studies of Negro-Vilar, 1978; Chihara et al. 1979; Vijayan, Krulich & McCann, 1975). The physiological role of such a pathway and that of a proposed serotonergic pathway remain obscure and the findings are controversial.

The secretion of adrenocorticotrophin (ACTH) is inhibited by a noradrenergic system in the brain. There is evidence that the neurons exert their influence via alpha-adrenergic receptors on the cells that secrete corticotrophin releasing hormone (Ganong, 1974). Acetylcholine has also been thought to be involved, but other data do not support this hypothesis (Ganong, 1974). Evidence is accumulating that the biogenic amines norepinephrine and epinephrine also have direct effects on the pituitary (Vale & Rivier, 1977; Giguere et al. 1981; Vale et al. 1983). Hence, the identity of the control mechanism remains uncertain.

For the role of neurotransmitters in the control of thyrotrophine secretion there is alas much contradictory evidence. Although there is in vivo evidence for an alpha-adrenergic control (Montoya, Wilber & Lorincz, 1979), the in vitro studies indicate stimulatory actions by norepinephrine, dopamine, serotonin and histamine (Jackson & Lechan, 1983).

### 1.2.6 Extrahypothalamic occurrences of releasing hormones

It is now recognized that the distribution of the releasing hormones is not restricted to the hypothalamus or even to the brain. They are also found in completely unrelated organs, where they apparently perform biological actions quite different from their hypophysiotrophic actions. E.g. TRH has been found to stimulate central nervous system motor activity (Segal & Mandell, 1974) and to modify autonomic nervous activity, to increase the plasma content of catecholamines and to increase heart rate and blood pressure (Yarbrough, 1979). CRH decreases parasympathetic stimulation in the intestines (Tache et al. 1983) and the heart (Fisher & Brown, 1983). It also increases peripheral norepinephrine sympathetic actions as well as the secretion of epinephrine from the adrenal medulla (Brown et al. 1982). In addition it can increase blood pressure, heart rate and hepatic glucose production (Fisher et al. 1982; Brown et al. 1982). Growth hormone inhibiting hormone inhibits epinephrine secretion (Brown, Rivier & Vale, 1981).

LRH has effects on the central nervous system in that it is capable of increasing libido after intraperitonal injection (Moss et al. (1973), which effect is not dependent on the pituitary (Pfaff, 1973). Administration of anti-LRH sera (Kozlowski and Hofstetter, 1978) and inhibitory LRH-analogues can suppress mating (Moss & Dudley, 1981). The releasing hormone has also been found in the mid-brain, a region known to be important in the control of mating behaviour (Samson et al. 1980). Furthermore, LRH has been detected in olfactory pathways in the hamster, and the olfactory sense is known to play a critical role in reproductive behaviour in this species (Philips et al. 1980).

LRH has also been found outside the nervous system, as large quantities were reported in placental cell cultures and in placental extracts (Siler-Khodr & Khodr, 1978; Khodr & Siler-Khodr, 1980; Gibbons, Mitnick & Chieffo, 1975). The hormone behaves in a manner indistinguishable to synthetic LRH in bioassays, radioimmunoassays and physico-chemical assays. Milk is another source of extrahypothalamic LRH. Baram et al. (1977) isolated a substance which showed LH-releasing activity in vitro, cross-reacted in a radioimmunoassay and co-migrated with LRH in chromatographic analysis.

Receptors for LRH have been identified in peripheral organs such as ovaries (Clayton, Harwood & Catt, 1979) and testes (Bourne et al. 1980). The hormone itself was detected by Paull et al. (1980). Evidence for direct luteolytic effects by LRH has been obtained (MacDonald, Greeley & Beattie, 1980), testicular steroidogenesis can also be inhibited by LRH, albeit only with pharmacological doses (Hsueh & Erickson, 1979).

It is obvious that the group of peptides known as hypothalamic releasing hormones have in fact many more effects and sites of actions than was originally thought. It is not very difficult to explain the the distribution of LRH in a teleological manner. It is concerned with reproduction on a number of levels: mating behaviour, gametogenesis, ovulation, gonadal steroidogenesis, placental function and neonatal sex differentiation. It might, therefore, be considered as a universal "reproduction hormone".

### 1.2.7 Nomenclature of releasing hormones

The releasing hormone nomenclature is still rather confused. Luteinizing hormone releasing hormone is for instance referred to as: luteinizing hormone releasing factor, acronym "LRF"; luteinizing hormone releasing hormone, acronyms "LRH" or "LH-RH" or "LHRH"; luteinizing hormone/follicle stimulating hormone releasing hormone (in view of its dual action), acronym "LH/FSH-RH"; gonadotrophin releasing hormone, acronyms "GnRH" or" Gn-RH"; with recommended names "gonadoliberin" (IUPAC-IUB) and "gonadorelin" (WHO).

Many commonly occurring peptides are known under trivial names, indicating either the source (e.g. insulin) or the physiological action (e.g. prolactin). Others are known by acronyms or abbreviations of overly long names (e.g. FSH). In dealing with hypothalamic releasing hormones it became common practice to call these substances "releasing factors" until their chemical identity could be established, whereafter they are referred to as "releasing hormones". As it was rather cumbersome to pronounce these long names, it quickly became a habit to use acronyms. The trivial names proposed by the above organizations have not reached common acceptance. The most common acronyms for luteinizing hormone releasing hormone are at the moment LHRH and GnRH but LRH and LRF are also used. At the time when these studies were carried out, LRH was very common. This, and the fact that a three-letter acronym is easier to use and pronounce than a four-letter one, led us to use the term LRH throughout this thesis.

# CHAPTER 2

## Fundamental aspects of radioimmunoassay

### 2.1 Introduction

One of the most urgent needs in the field of hormone research is the development of techniques of sufficient specificity and sensitivity to enable measurements to be performed in biological fluids at short time intervals and in small amounts of material. This is necessary in order to study acute physiological changes in hormone levels.

For the assay of biologically active substances we have, in addition to physico-chemical methods, two general types of techniques based on biological principles : the bioassay and the ligand-binding assay (the latter is also known as saturation analysis). A bioassay utilizes a biological effect of a hormone in order to compare unknown samples with standardized quantities of the biologically active substance. A binding assay estimates a substance on the basis of a specific binding characteristic of the whole molecule or a part of it. The ligand is usually a polypeptide derived from a biological system and may be an antigen, a receptor protein or any other compound, capable of specifically binding the substance be determined.

In this study we shall deal with a special type of binding assay, the immunoassay, where the ligand is an antibody. More specifically, we use a radioimmunoassay, where the measured parameter used for calculation of results is the degree of displacement from binding of a radiolabeled antigen by its non-labeled counterpart.

### 2.2 Advantages and disadvantages

Radioimmunoassays offer several advantages over the earlier determination methods such as bioassays. High sensitivity is the most important advantage, a radioimmunoassay, at least in theory, permitting the determination of quantities as small as femtomoles (0.000000000000001 moles). High specificity is a second advantage as they offer an excellent ability to measure only the desired compound. High precision and reproducibility, even at very low concentrations of the substance to be determined, is a third important advantage of radioimmunoassays. These advantages can only be obtained when certain conditions are met and certain limitations are accepted. In developing an assay, it is necessary that it be thoroughly validated

according to established principles. Especially, there is a need for extensive characterization of the antibody. It must be recognized that the radioimmunoassay does not necessarily measure the active principle as the case in a bioassay, since the antibody may also react with a biologically inactive precursor or metabolic derivative of the compound to be measured. Moreover, production of antibodies is a time-consuming and laborious technique, a supply of properly prepared and characterized antibody, once obtained, can be used for many years of work. It is not uncommon to be able to do millions of determinations from a single bleeding of a rabbit (Berson & Yalow, 1976).

## 2.3 Basic principles of the radioimmunoassay

A radiolabeled antigen was first used to study the interactions of an antigen and its antibody in vitro. This eventually led to the demonstration of circulating antibodies to insulin in humans who were treated with insulin derived from other species (Berson et al. 1956). Berson & Yalow (1958) were the first to point out the remarkable sensitivity obtained when unlabeled antigens are measured by their ability to inhibit competitively the binding of radiolabeled antigens by antibodies. At approximately the same time Ekins studied the binding of radiolabeled vitamin B12 to intrinsic factor. These studies led to the development of the first radioimmunoassays by Yalow & Berson (1959 and 1960) and Ekins (1960). The basis for the radioimmunoassay is shown in the following reaction scheme:

| free labeled antigen | + | anti-body | + | unlabeled antigen | <=> | labeled antigen-antibody complex | + | unlabeled antigen-antibody complex |
|---|---|---|---|---|---|---|---|---|
| $Ag^*$ | + | Ab | + | Ag | <=> | $Ag^*$-Ab | + | Ag-Ab |

In performing the assay, labeled antigen and the specific antibody, are incubated in the presence and absence of a sample, containing the unknown amount of (unlabeled) antigen. After equilibrium has been reached or approached, free and antibody bound antigen are separated by a suitable physico-chemical method. The radioactivity of one or both (usually one) of these fractions is measured. The antigen concentration in unknown samples is calculated by comparing the decrease in binding of labeled antigen produced by the unlabeled antigen in the sample, with that of a standard curve obtained by adding known amounts of unlabeled antigen to the assay system.

## 2.4 Requirements for radioimmunoassays

The requirements for any radioimmunoassay are: a purified antigen, a standard preparation of the antigen, a specific antibody and a method for separating the bound and free antigen fractions. In addition, extraction and chromatographical steps may be required, depending on the nature of the sample and the possibility of interference by closely related compounds or by non-specific factors.

A supply of purified antigen is an essential prerequisite for the development of a radioimmunoassay. Thus the application of a radioimmunological technique is limited to substances, where this criterion can be met. If the pure substance is available, as is often the case with steroid and peptide hormones, this substance is used for antibody induction, preparation of radiolabeled antigen and for standards. If the substance itself not immunogenic, it can usually be made so by a suitable conjugation.

It should be noted, however, that a pure preparation is not absolutely required for use as a standard. A partially purified antigen, or even a biological material with a high content of the substance that is to be determined, can be utilized. E.g. it is possible to use a pool plasma standard in a radioimmunoassay (Berson & Yalow, 1966). Obviously, in this case, the results will not give molar concentrations. It is then necessary, in addition to standard validation procedures, to calibrate the method against an International Reference Preparation or similarly characterized and accepted material.

Partially pure antigen preparations can also be used for immunization, since the resulting antibodies must in any case be selected by means of extensive characterization. Experience shows that a specific antibody may result after immunization with a very pure antigen as well as with a mixture of substances (Yalow, 1973). Given a choice, it might be better to use the pure antigen.

For the preparation of the labeled antigen it is vital to use a highly purified substance. The immunological integrity of the labeled antigen is of crucial importance, since the performance of the assay will greatly depend on its purity and its identity with the substance to be measured. Since labeling of the antigen usually involves its chemical alteration, it is necessary to purifiy the reaction product, e.g. with chromatography, and then check the purified substance for its binding capacity to the antibody. For the same reasons, a labeling method, causing minimal damage to the antigen, should be selected. It might be possible to purify the labeled material after completion of the reaction, but this is not always feasible. Chapter 3 supplies details of the preparation of radioiodinated LRH.

In addition to its dependence on tracer quality, the performance and reliability of a radioimmunoassay will depend above all else on the properties of the antibodies. Three aspects are of importance: avidity, specificity, and availability. The avidity of the antibody will largely determine the maximal sensitivity of the assay, which may be of crucial importance. The necessity of employing an antibody with the highest possible specificity is obvious. Availability of the antibody involves the ease or difficulty of raising the antibody, the reproducibility of its characteristics in successive bleedings, and the stability of the antibody during storage. Production of anti-LRH sera is discussed in Chapter 3, the determination of their specificity in Chapter 5.

In order to measure the fraction of labeled antigen that at a given time is in the free and the bound form, it is necessary to physically separate the two fractions. This can be achieved by any of several methods, which utilize a physico-chemical difference between the two fractions. E.g. the addition of an organic solvent to the incubation medium may often precipitate the antibody-bound antigen but not the free antigen. The main criteria for the separation methods are efficiency and practicality. The choice of a separation method for the LRH-RIA is discussed in Chapter 4.

## 2.5 Optimization and validation of radioimmunoassays

Optimizing of the assay conditions usually has to be accomplished in an empirical fashion, but the problem can also be approached in a mathematical way. Several investigators have attempted to mathematically define optimum conditions for radioimmunoassay (Ekins & Newman, 1970; Ekins, Newman & O'Riordan, 1971; Berson & Yalow, 1971 and 1976; Rodbard & Lewald, 1970; Rodbard, 1971; Ekins, 1974). They have reached somewhat different conclusions, especially with regards to the optimal concentration of reactants. Nevertheless, it seems safe to state that in order to obtain maximal sensitivity of the assay, low concentrations of the antibody must be chosen while still maintaining practical assay conditions. The limiting factor here is that by lowering antibody concentration, we increase the relative amount of non-specific binding in the measured radioactivity, thus burying the gain in sensitivity at the expense of the precision. Preincubation of the unknown sample with antibody prior to addition of the labeled antigen might be advantageous for high sensitivity. In the case of the LRH-RIA these factors are discussed in Chapter 4.

In order to ensure optimal sensitivity, the antigen must be maximally labeled so as to yield a high specific activity. The reason for this is that the labeled antigen should be present in minimal concentration, while still giving the highest possible counting rate

(Yalow & Berson, 1969 and 1971). The limiting factor here is the decreasing level of precision as the level of radioactivity decreases (Ekins, 1974). In developing a radioimmunoassay it is advisable to check out these effects. More important is that the integrity of the antigen be preserved during labeling procedure. See also Chapter 3 for the preparation of I-125 labeled LRH and Chapter 4 for the influence of the tracer concentration on the behaviour of the LRH-RIA.

The choice of the incubation temperature may also be of importance, since the sensititvity of the assay will be maximal by incubating at the temperature at which the association constant of the reaction is highest. Should this be at a low temperature, then a longer incubation time is required in order to achieve sufficient binding of antigen to antibody. The volume of the incubation mixture also plays a role in that elimination of unnecessary water has an effect similar to that of changing to a more avid antibody, which means that the assay becomes more cost-effective. These factors are investigated in Chapter 4.

As pointed out in section 2.2, the nature of the radioimmunoassay technique makes it imperative to investigate exactly what is being measured. One obvious point to consider is the question of antibody specificity. Antibodies are usually directed to only a part of a molecule, and thus depending on the circumstances, degradation products, precursors, and other structurally related molecules may be measured as the original molecule. The immunological specificity can be investigated by testing analogues of the antigen for their ability to displace labeled antigen from binding to the antibody. Chapter 5 present our data on the specificity of anti-LRH sera.

One should also ascertain the immunological identity of the standard preparation and the sample material. One way of doing this is to check whether they give parallel dose-response curves. This implies that it is desirable to assay on at least two, and preferably more levels of sample dilution. For the LRH-RIA this is presented in Chapter 7. It is also desirable to compare the radioimmunoassay with another, completely different, type of assay which has been established and validated, e.g. a bioassay or, if possible, a reliable analytical technique such as mass-spectrometry-gas-chromatography. For the LRH-RIA we have done this by comparing with a standardized bioassay, which is reported in Chapter 5.

## 2.6 Antibodies

Antibodies are the key components of all radioimmunoassays. The specificity and, to a large degree, the maximally possible sensi-

tivity of the assay will depend on the quality of this reagent. Their production must be provoked in such a manner, that the resulting antibodies are of the required standard. The likelihood of obtaining suitable antibodies is dictated largely by chance, but a number of factors influence the success rate. This includes the choice of proper immunization schedules, antigen and injection technique.

### 2.6.1 Immunogenicity of proteins and small peptides

All proteins are immunogenic, although not to the same extent and usually not in the species in which they arise. The factors that confer immunogenicity are complex and incompletely understood. However, it is known that certain criteria must be satisfied. In general, the immunogenicity of a molecule is directly related to its size. A molecule with a molecular mass above 10,000 usually provides a relatively easy production of antibodies, whereas molecules with a mass of less then 1000 are poor immunogens. Obtaining antibodies to molecules between these two limits meets with variable succes.

However, size is not the only factor playing a role. For a protein to elicit an antibody reaction it must normally be recognized by the organism as foreign. It has been observed by Fleischer et al. (1965 & 1966) that in the case of phylogenetically related molecules the antibody will be directed to those parts of the molecule that are different. It also seems that greater chemical complexity will make the molecule more strongly immunogenic. The method of administration of the antigen is also of great importance. Intravenous injection of a compound may, for instance, not lead to any antibody production, whereas subcutaneous injection with an adjuvant may lead to copious antibody production (Orth, 1975).

Finally, it is possible to enhance, or indeed induce, the immunogenicity of a molecule by chemically altering it. Virtually any chemical entity may serve as an antigenic determinant if it is coupled to a suitable carrier. In fact, by means of chemical alteration it is possible to produce antibodies even against metal ions (Goodman, 1976).

Because of the uncertainty whether a peptide with a molecular mass between 1 - 10 kD, such as LRH (molecular mass = 1128), will produce antibodies, it is advisable to conjugate it to a protein before immunization. Many investigators, who started out using unconjugated peptides, have subsequently switched to the use of conjugates. E.g. Orth (1975), who had to use more than 200 animals to obtain a single antiserum from unconjugated ACTH, could obtain antibodies almost immediately when using an albumin conjugate.

2.6.2 Conjugation of a small molecule to a carrier protein.

In a case, as in the present (LRH), when the immunogenicity of the compound is thought to be insufficient, it is common practice to bind the small molecule covalently to a carrier, usually a protein. The carrier protein can be a homologous polyaminoacid (Vunakis, 1971) or a naturally occurring protein such as serum albumin (Niswender & Midgley, 1970), thyreoglobulin (Bauminger, Kohen & Lindner, 1974) or haemocyanin (Doerr & Crambach, 1971). The characteristics of the resulting antisera will depend on the nature of the small molecule, the nature of the carrier protein, and the way in which they are coupled.

A number of reactions are available for the conjugation procedure. The most common one is the carbodiimide reaction (Khorana, 1953; Goodfriend, Levine & Faisman, 1964). In this reaction a carboxyl group of the small or large one molecule is linked to an aminogroup of the other molecule to form a peptide link. Either the small molecule or the carrier molecule can provide the carboxyl group and vice versa the aminogroup. Another procedure is the mixed anhydride reaction according to Boissonas (1951) and Erlanger et al. (1957). In this case a carboxylic function on the small molecule is activated to form an anhydride, which can then be aminated by a free amino group of the carrier protein. The glutaraldehyde reaction can be used to couple two amino groups, present on the small molecule and the carrier protein respectively (Reichlin et al. 1968, Richards & Knowles, 1968). When no carboxyl groups are available, as in the case of steroids, a hydroxyl group can be esterified with succinic anhydride (Mikhail et al. 1970), or an oxime derivative formed by the reaction with o-carboxylmethyl hydroxylamine. In both cases the modified molecule can then be linked to an amino group of a carrier protein, e.g. bovine serum albumin (Erlanger, 1973; Liebeman et al. 1959). The classical diazo reaction can be used to bind either a histidine or a tyrosine moiety of the carrier protein to a diazo salt of the small molecule (Kopitar & Kompa, 1975; Farai et al. 1975). Finally it is possible to use the bis-diazotized benzidine method for the same purpose. This method has been used extensively for small peptides like thyrotrophin releasing hormone (Likhit & Sehon, 1967; Bassiri & Utiger, 1972).

2.7 Labeling of the antigen

An essential aspect of any radioimmunoassay is the determination of the distribution between bound and free fractions of antigen. For this purpose a fixed amount of a labeled ligand (here: antigen) is added to the reaction mixture. The label may be any substance with a

characteristic property that can be measured reliably and simply. In a radioimmunoassay this is a radioactive isotope. The label can be introduced by a variety of methods, depending on its physical and chemical nature.

Basically we can distinguish between two types of radiolabeling: internal and external.In the case of internal labeling, an existing atom in the antigen molecule is replaced by a radioactive isotope e.g. carbon-14 or tritium. The great advantage of the internal labeling method is that the labeled antigen is chemically identical to the unlabeled one present in samples and standards. External labeling involves the introduction of a radioactive isotope by covalent linkage to an existing structure in the antigen molecule. Although such a molecule is per definition not identical to the unlabeled antigen, in practice its immunological characteristics are often indistinguishable from those of the unlabeled antigen.

Tracers with internal label are often commercially obtainable and are usually prepared by one of four basic techniques: neutron irradiation, chemical synthesis, biological synthesis or exchange reactions. Neutron irradiation is rather unselective and can lead to damage of the molecule being labeled. Chemical synthesis involves the use of straight forward synthesis but using a labeled component. Biological synthesis follows the same principle, but using a biological system such as an intact (micro-) organism or an enzyme system rather than a chemical system. Exchange reactions are commonly used for tritium labeling. The material to be labeled is kept in contact with tritium for a prolonged period of time, during which an exchange between hydrogen and tritium will take place.

External labeling of peptide antigens is most commonly carried out with radioactive iodine, since iodine substitutes relatively easily into the aromatic side-chain of tyrosine. Histidine also reacts with iodine, but much slower than tyrosine. While in the early days of radioimmunoassay 131-iodine was used, The present availability of 125-iodine with high specific activity has made it the isotope of choice because of its longer half- life.

### 2.7.1 Methods of radioiodination

A number of methods have been described for introducing iodine into peptides. They all have in common that the relatively unreactive iodide is converted into the more reactive iodine, which can then be incorporated into tyrosine residues of the peptide to be labeled (Ramachandran, 1956). The older techniques, viz. the iodine monochloride method according to MacFarlane (1958) and the electrolytic

method (Rosall, 1964), are hardly used anymore. A more common technique uses Chloramine T, an oxidizing agent which converts iodide to iodine simply by mixing the two substances (Hunter & Greenwood, 1962; Greenwood, Hunter & Glover, 1963). The reaction is stopped by adding the reducing agent sodium metabisulphite. A hazard of the method is the possibility of damage to the peptide by the oxidative process. The level of iodination can be controlled by varying the concentration of the oxidant and the length of exposure. Both should be kept to a minimum consistent with a sufficiently high radioiodination level in order to avoid nonspecific damage by Chloramine T (Yalow & Berson, 1971). The simplicity of the method explains its almost universal acceptance. Purification is carried out immediately in order to remove unreacted iodine and unwanted reaction products. Standard biochemical methods, such as chromatography on molecular sieves (e.g. Sephadex gels) are used for this purpose.

Enzymatic oxidation has the advantage of not exposing the peptide to relatively high concentrations of a chemical oxidizing agent. Lactoperoxidase in the presence of traces of hydrogen peroxide is used to introduce the radioiodine into the peptide (Marchalonis, 1969, Thorell & Johansson, 1971). A reducing agent is not required, since simple dilution of the reaction mixture will stop the reaction. The iodination mostly occurs in tyrosine residues, although as with Chloramine T iodination of histidyl groups is also possible. It has been claimed that this procedure will result in less damage to the labeled substance than other methods (Thorell & Johansson, 1971; Karonen et al. 1975).

Conjugation labeling, as described by Bolton & Hunter (1973), offers several advantages. With this technique the radioiodine is first coupled to a phenol or imidazole group of an appropriate carrier containing an amine group for coupling to the antigen. Radioiodination of the carrier molecule can be executed with any of the previously described methods, whereupon the labeled carrier is coupled to the antigen by means of a standard conjugation reaction. This way of labeling has the advantage of not exposing the peptide to any possible chemical damage of the oxidizing reaction. it can be applied to peptides without tyrosyl or histidyl residues. A disadvantage of the method is that the label is much larger and may thus lead to alteration of the immunological behavior of the labeled antigen.

### 2.7.2 Non-radioactive labels

Radioactive decay is, by definition, a singular event and only a small fraction of all radioactive atoms in a sample are in fact emitting radiation during a typical observation period. On the other hand,

other probes such as discussed below, can be used in such a manner that virtually all molecules will undergo reaction in a limited time and thus provide a far more efficient system. Consequently, such methods might yield more sensitive assays, especially if used in techniques such as immunoradiometric assays.

A common non-radioactive labeling method is the use of enzymes instead of radioactive substances (van Weenen and Schuurs, 1972; Wisdom, 1976; Schuurs and van Weenen, 1977; Engvall, 1980). Because of the catalytic nature of enzymes a small quantity of label can be measured relatively easily by means of conventional and well established laboratory methods. The advantages of enzyme-immunoassays are a prolonged shelf-life of the labeled antigen, absence of radiation damage to the labeled antigen, absence of radiation hazards in the laboratory and that expensive counting equipment is not needed. A possible source of error with this technique is noise due to endogenous enzyme activity in the samples. The sensitivity of the assay will depend on the source of the label, ultimately on the number of molecules being catalytically converted and the method employed to detect this conversion. Standard fixed-time spectrophotometric determination method are commonly used. The more rapid kinetic type of assay is less commonly used since most enzyme immunoassays are employing a solid phase separation of free and bound antigen, which leads to turbid solutions and hence to difficulties in reading initial reaction rates. In such cases the use of specific electrodes capable of detecting e.g. ammonia or oxygen can be used to determine the reaction rate (Meyerhoff & Rechnitz, 1980).

A variant of the use of enzyme labels is the prosthetic group label immunoassay (Morris et al. 1981). This is in fact a sort of homogeneous immunoassay (see also section 2.8) where advantage is taken of the fact that an antigen-prosthetic group (FAD) conjugate is able to activate (apo-)glucose oxidase, but is strongly inhibited when the conjugate is bound to the specific antibody. A conventional detection system for the oxygen consumed or the hydrogen peroxide liberated by the oxidase is used to quantify the reaction rate. This could be the classical Trinder colour generating system (Barham & Trinder, 1972) or any other convenient system available such as an oxygen sensitive electrode.

Another approach for the immunoassay is the use of electron spin resonance (Leute et al. 1972). The antigen is then coupled with a spin label like nitroxide, which has a characteristic sharp-banded electron spin resonance spectrum. The change in the spectrum upon reaction of the antigen with its antibody is observed. The method is simple and rapid, but requires expensive equipment and with the present instrumentation the lower detection limit is unfortunately several orders of magnitude higher than that of the radioimmunoassay

technique.

Yet another possible labeling method is the use of a fluorescent molecule such as fluorescein (Aalbers, 1973; Bryce, 1974). These assays share a disadvantage with the enzyme immunoassay, viz. interference by endogenous (here: fluorescent) compounds in samples, also the fluorescence arising from plastic or glass containers. When an antigen conjugated to fluorescin is reacted with its antibody, a change in fluorescence polarization can occur (Parker, 1973). Alternatively, fluorescence quenching can be induced by the binding and the reduction in fluorescence used as a parameter for the detection (Dandliker, Hsu & Vanderlaan, 1980). It is also possible to use techniques such as the fluorescence fluctuation method for the same purpose (Elings, Nicoli & Briggs, 1983). The search for superior fluorophores led to the use of chelated compounds of rare earth elements, e.g. europium. These materials display very characteristic fluorescence, including a prolonged decay fluorescence, which permit discrimination between label-induced photons and background fluorescence (Ekins, 1983).

The above mentioned europium chelates is but one example of the use of metal labels in immunoassay. In fact, metals can generally be used as labels in place of radioactive isotopes. The detection of the metal may be carried out by any of a number of more or less conventional methods such as emission, absorption and fluorescence spectrometry as well as microwave excitation emission spectrometry, anodic stripping voltammetry and neutron activation (Cais, 1983). The metals are either incorporated directly in the antigen forming organometallics or are introduced as part of a molecule that is attached to the antigen.

A chemiluminescent label offer potentially very high effective specific activities (Ekins, 1983). This involves the use of compounds capable of emitting light as a result of chemical reactions. A special case is the use of bioluminescence, which allows for a very sensitive assay (Wannlund & DeLuca, 1983). The measured parameter in this system is light emitted from a reaction catalyzed either by firefly luciferase or bacterial luciferase. In the first example the luciferase is coupled to the antigen in a manner allowing it to retain its enzymatic activity and then this label is used in a conventional immunoassay. Alternatively the antigen is coupled to glucose-6-phosphate dehydrogenase and the NADH produced is detected by adding a bacterial luciferase NADH-dependent light emitting enzyme system.

## 2.8 Variants of radioimmunoassay

Many proteins have been identified, which have an affinity for molecules such as steroids (Korenman, 1968), vitamins (Brumbaugh et al. 1974), hormones (Catt et al. 1971) and other biologically important compounds. These proteins can be used for competitive binding assays in a manner resembling the radioimmunoassay. Many binding proteins are found in blood, where they serve for transport or storage of molecules such as corticoid steroids (Murphy, 1971) or sex steroids (Mayers & Nugent, 1968; Johansson, 1969). Other binding proteins occur in cells, either in the plasma membrane serving as a receptor for hormones regulating events inside the cell via a second messenger system, or in cytoplasm or nucleus and having affinity for molecules acting within the cell like e.g. cyclic AMP (Gilman, 1970). Although immunoassays and binding protein receptor assays may vary considerably in sensitivity and specificity, the one system is not always superior to the other. The two types of methods suffer indeed from the same pitfalls and, by and large, have the same precision and reproducibility.

A very common variant of the radioimmunoassay is the enzyme-immunoassay. This can be an assay where the radioactive label on the antigen or antibody is replaced by an enzyme (van Weenen & Schuurs, 1972). The rest of the assay, e.g. the separation of bound and free antigen, is executed in the same manner as for the radioimmunoassay. Like the radioactive label, the enzyme marker can be attached to either the antigen or the antibody. However, there is a fundamentally different type of immunoassay employing enzymes: the so called homologous enzymeimmunoassay (Rubinstein, Schneider & Ullman, 1972). Here the enzyme activity of the label is inhibited upon binding to the antibody and so the remaining activity will reflect the amount of competing antigen in the sample. As the technique does not require a separation of bound and free fractions and is therefore less laborious and can easily be subjected to automation with common clinical chemistry analyzers.

An important variant of the radioimmunoassay is the immunoradiometric assay. In this system one labels the antibody rather than the antigen (Miles & Hales, 1968). As the principal antibody protein, immunoglobulin G; contains many tyrosine residues, it can be iodinated relatively easily without loss of antibody activity. The radioimmunometric assay is advantageous where it is difficult to produce a labeled antigen, e.g. due to the lack of tyrosine residues in a peptide. Alternatively, in some cases one might wish to avoid handling the antigen, as in the case of hepatitis B antigen. It has the additional advantage that a standard procedure can be set up for all labelings in a laboratory. A perhaps more important aspect is that this type of assay can be made more sensitive than any other immunoassays (Ekins, 1983).

This presupposes the use of labels with a higher intrinsic maximal specific activity than radioisotopes. This can be done by using e.g. chemiluminescent or fluorescent probes. The immunoradiometric type of assay requires relatively large amounts of antibodies. It might therefore be of advantage to use monoclonal antibodies, which can be produced in almost unlimited amounts of uniform and relatively pure product. Hence, this type of assay may find a multitude of applications in the future.

# CHAPTER 3

## Production of reagents for the radioimmunoassay

### 3.1 Antibodies

When our studies were initiated, LRH was available only in very limited quantities and only to specially invited research groups. The author was privileged to receive generous supplies of the LRH decapeptide from Dr. F. Enzman of Hoechst AG, Frankfurt, W.Germany. However, although at one time we received half of the entire supply present in Europe at that moment, only milligram amounts of the compound was available to us. This had, of course, a decisive influence on the choice of immunization techniques and immunogens. The Hoechst company was interested in having radioimmunoassays for LRH established and hence prepared LRH conjugates for us and for other investigators use. Consequently, the immunization schedules were made to fit the available synthetic materials. This meant that at times when only the unconjugated LRH was available, the pure LRH was used as immunogen.

This was done on the, perhaps dubious, theory that antibodies might develop upon challenge with the pure substance in an animal that had already been exposed to the more immunogenic conjugate. The alternative would have been to undertake the conjugation of the scarce LRH ourselves. We choose not to do this, as we were aware of the poor results obtained by many laboratories in several countries, where considerable experience and sophisticated techniques were available.

As it turned out, we had perhaps been unnecessarily pessimistic. At that time a graduate student in Scotland attempted to prepare a LRH-BSA conjugate with the carbodiimide method of Goodfriend et al. (1964). According to theoretical considerations, discussed in section 3.3, this should not be possible. However, apparently he was unaware of this and succeeded! The resulting conjugate was injected into two rabbits and he could harvest, apparently almost effortlessly, an antiserum well suited for the radioimmunoassay of LRH (Fraser & Gunn, 1972). Because of its simplicity and high rate of success, the method was adopted by us and others and led to the production of antibodies to LRH (Seppaelae, Ranta &. Leppaeluoto, 1974; Burger & Franchmont, 1974; Root et al. 1975; Kizer et al. 1976; Dahlen et al. 1976).

We used as immunogens three conjugates of LRH, viz. to polylysin, cellulose and bovine serum albumin, in three separate attempts.

All conjugates, and synthetic LRH, were prepared and purified in the Hoechst AG laboratories and were made available to us at different times. The substances were dissolved in saline and homogenized in an equal volume of Freund's complete adjuvant. The suspensions were injected in volumes of 50 - 100 µl per site in the quantities stated in the following three paragraphs. Injections were done intradermally at 60 - 100 sites on the shaved backs of six male New Zealand white rabbits for each conjugate. Initially we gave the booster injections subcutaneously in Freunds' incomplete adjuvant. In later studies we have also used the complete adjuvant and intradermal multiple site injections for the booster injections. Blood was withdrawn by puncturing one ear vein and placing the ear in a partly evacuated container. In this manner 30 - 50 ml blood could be collected in a few minutes with a minimum of discomfort for the animal. Serum was collected by allowing the blood to clot at 4°C for 18 - 24 hours and centrifuging it to remove the clotted material. The serum was collected and divided into 0.1 - 1.0 ml aliquots that were snap-frozen by immersion in an ethanol bath at -40°C.

Polylysin-conjugated LRH was given to a first group of six rabbits. Two animals received 4 mg of the conjugate, two others 3 mg, and the remaing two 2 mg. Seven weeks after the preliminary injections 1 mg unconjugated LRH was administered (at this time no conjugated LRH was available) in an attempt to boost the effect of the initial injections. Serum was collected on five occasions at monthly or bimonthly intervals. No anti-LRH activity could be detected in any of the sera.

Cellulose-conjugated LRH was given to another group of six rabbits. The primary injection of 0.8 mg conjugate per rabbit was followed by a booster injection of 1 mg unconjugated LRH on the 9th week of the injection schedule for the same reasons as above. No anti-LRH activity could be found in any of the sera obtained over a 6-months period after immunization.

A succesful induction of LRH antibodies was achieved with LRH coupled to bovine serum albumin according to Frazer & Gunn (1973). Each of the six rabbits received 3 mg conjugate and a booster schedule was initiated after 4 months consisting of biweekly administration of 0.2 mg conjugate over a two-month period. Three rabbits developed antibodies to LRH in response to this treatment, but only one produced concentrations suitable for radioimmunoassay. All three rabbits received further booster injections, but only the rabbit with the high titre continued to produce suitable antiserum. Specificity studies indicated that the antibodies were of high quality, so no further attempts were made to produce antibodies.

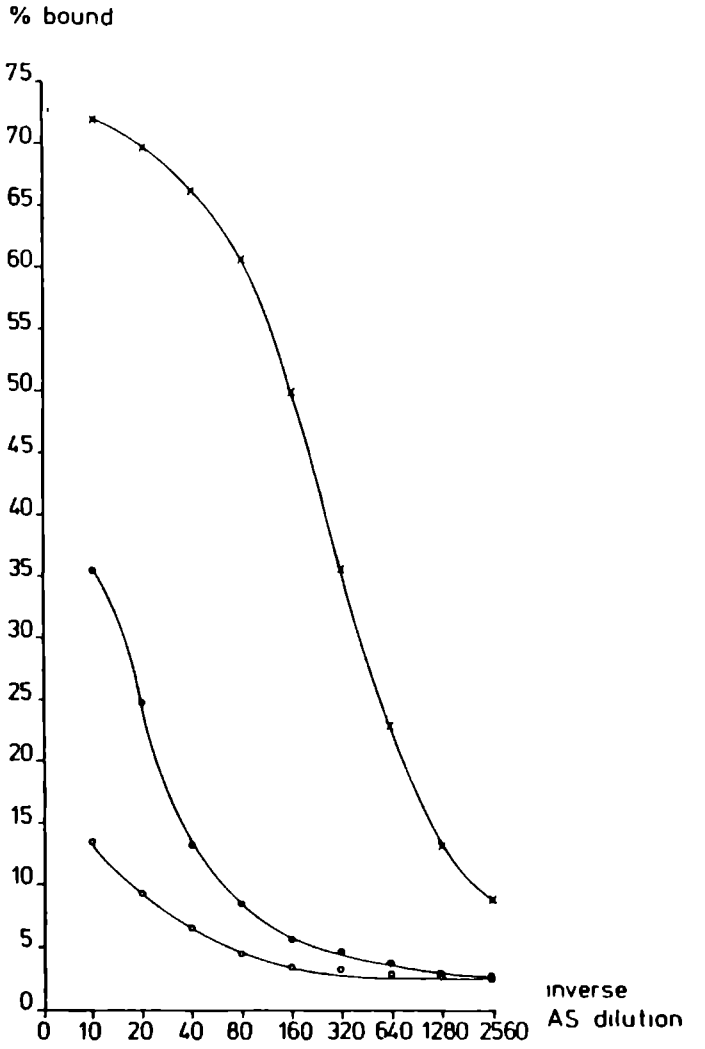


**Figure 3.1 : Antiserum dilution curves for three LRH antisera produced by incubation with a fixed amount 125-I-LRH and separation of bound and free antigen by means of solvent precipitation.**

We tested for the presence of anti-LRH activity in the rabbit sera by incubating serial dilutions of the sera with a fixed amount (approx. 10,000 cpm) of radio-iodinated LRH (for its preparation, see

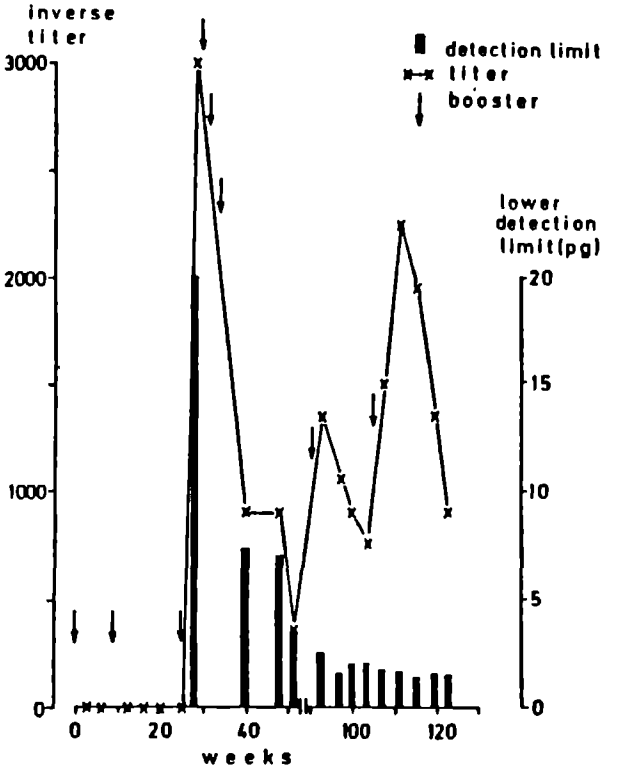


Figure 3.2 : Antibody titre and assay sensitivity for different bleedings from an anti-LRH producing rabbit

section 3.2). The incubation lasted 3 - 4 hours at 4°C, which is enough to reach near-equilibrium conditions (see section 4.2). Bound and free antigen were then separated by means of ethanol precipitation of the bound fraction. This was the method of choice for the LRH radioimmunoassay (see also section 4.3). Centrifugation and measurement of radioactivity in the precipitate in a LKB-Wallac gamma-counter 8000 completed the test.

Figure 3.1 shows typical dilution curves for serum obtained from the three antibody producing animals. The uppermost curve represents the only antiserum suitable for radioimmunoassay. This rabbit produced good antisera for an extended period of time. The booster and bleeding schedules are presented in figure 3.2. This figure illustrates how the antibody titre varied in response to the booster injections. The antibody titre is defined as the dilution at which half of a standard amount of radioiodinated LRH (see section 3.2) is bound to

the antibody. Also shown in the figure is how the sensitivity of the assay varied when sera from different bleedings were used. The lower detection limit was defined as the amount of LRH needed to produce a significant reduction in the amount of radio-iodinated LRH bound to antibody in the absence of added standard LRH (see section 4.4 for details).

3.2 Preparation of the radioiodinated hormone

LRH contains a tyrosine residue in position 5, hence we chose radioiodination by means of the Chloramine T method. Through a trial and error approach the following method was established: To 5 µg pure synthetic LRH in 10 µl bidistilled water (predispensed in 1 ml serum vials and kept at -30°C) we add 25 µl 0.5 M phosphate buffer (pH 7.5) and 1 mCi 125-iodine in 5 µl aqueous solution as sodium Iodide. The radioiodine was purchased from New England Nuclear in volumes varying between 5 - 10 µl, in 5 mCi lots. Each lot was diluted to 25 µl to facilitate handling. The reaction is initiated by the addition of 20 µg Chloramine T dissolved in 10 µl 0.05 M phosphate buffer (pH 7.5). The content of the vial is mixed for 15 seconds on a Whirlmix, and the reaction is then stopped by the addition of 125 µg sodium metabisulphite dissolved in 50 µl phosphate buffer (0.05 M, pH 7.5). Then 500 µl carrier protein solution (5% egg albumin in phosphate buffered saline) is added to minimize losses from non-specific absorbtion on glass and other materials. The reaction products are separated by means of gel chromatography over a 15 x 300 mm Sephadex G-25 column, equilibrated with 0.01 M acetic acid, which also was used for elution. Fractions of 40 drops each are obtained on a fraction collector. The radioactivity of each fraction is measured on a gamma counter. We regularly find that 3 or 4 peaks of radioactivity are present, and that the last of these contains the material with the highest immunoreactivity. For routine use we pool the 3 - 5 tubes with the highest radioactivity in the last eluting component. The radiolabeled LRH is stored at -30°C in 0.1 ml aliquots. Radioiodinated LRH prepared in this way is stable for about 4 - 6 weeks. The immunological integrity as determined by binding to excess antibody was good to excellent: binding was never below 80 % and usually between 90 - 95%. Under standard assay conditions (see section 5.2.2) the non-specific binding of the tracer never exceeded 5% of the total radioactivity.

A more detailed characterization of the reaction mixture has been obtained by applying it to a longer Sephadex G-25 column. Fractions of 100 drops are collected and three consecutive fractions are pooled. The pooled fractions are diluted with phosphate buffered saline (0.01 M phosphate, 0.14 M sodium chloride, pH 7.0 with 0.1% egg albumin) so as to contain 100 cpm/µl. The immunoreactivity of

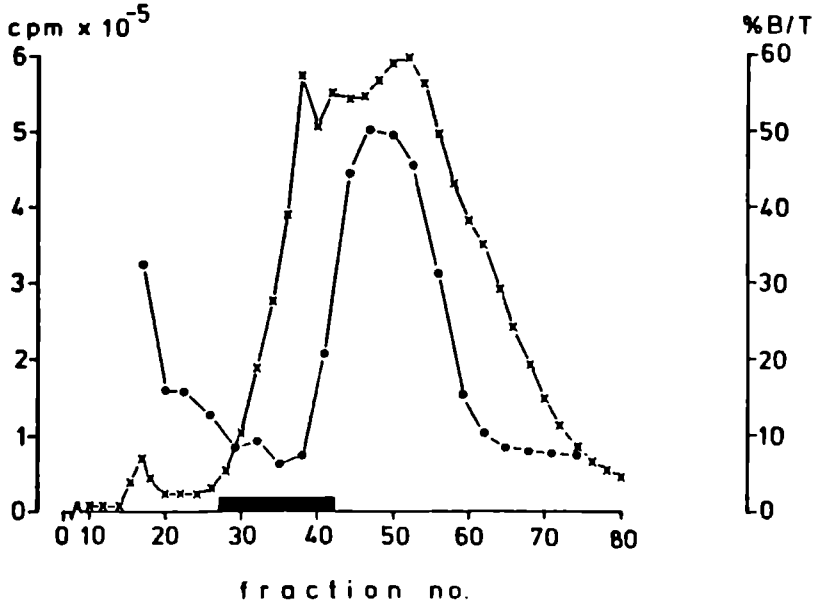


Figure 3.3 : Purification of 125-I labeled LRH on a Sephadex G-50 column, x---x denotes radioactivity and o---o denotes immunoreactivity expressed as % bound to antiserum under optimized conditions, the black bar represents immunoassayed LRH in the experiment with labeled LRH.

each fraction is tested by incubating 100 µl of the above dilution with 200 µl antiserum, appropriately diluted with the above phosphate buffered saline but containing 0.05 M EDTA and 0.25% normal rabbit serum. After incubation over night at 4°C bound and free antigen are separated by the addition of 5 volumes of cold ethanol and subsequent centrifugation at 1000 x g for 5 minutes. The behaviour of the radiolabeled LRH on the Sephadex column was further investigated by chromatography of 1 ng of pure synthetic LRH on the same column. Fractions collected as described for the radioiodination reaction mixture have a pH far too low for the antigen-antibody reaction. Hence, in order to determine LRH content of the fractions, we extract with ethanol before the radioimmunoassay. The extraction procedure is described in section 6.3 and the immunoassay procedure in section 5.2.2.

The results of the two experiments are summarized in figure 3.3. Surprisingly 125-iodine-LRH elutes after the unreacted iodine, rather than before as would be expected. Furthermore, the unlabeled

LRH elutes before or together with the iodide. This suggests that radioiodinated LRH is retained on the Sephadex G-25 material by a mechanism not related to difference in molecular size but rather to the presence of iodine in the molecule.

### 3.3 Discussion

Experience with the production of antibodies against small peptides was first obtained in work with the neuro-hypophyseal peptides and thyrotrophin releasing hormone. It was found that antibody production could be enhanced by rather elaborate techiques such as injection of the immunogen into the spleen or the lymph nodes (Boyd, Landon & Peart, 1967; Chard, Kitan & Landon, 1970). These methods were, however, soon replaced by the conjugation techniques and, above all, the very effective intradermal multiple site injection method of Vaitukaitis et al. (1971). The first report on the successful generation of anti-LRH sera by a group of French investigators (Kerdelhue et al. 1973a and 1973b) described the injection of pure synthetic LRH in guinea pigs for prolonged periods of time. The hormone was prepared in Freund's complete adjuvant and injected in or near the lymph nodes. At the end of the injection schedule the LRH was adsorbed to aluminium oxide before injection.

A conjugation method was used by Nett et al. (1973), who coupled the LRH-analogue pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-COOH to lysine residues of bovine serum albumin by means of the mixed anhydride reaction (Boissonas, 1951). They also used LRH coupled to bovine serum albumin by means of the bis-diazotized benzidine technique. Intradermal multiple site injections were made in rabbits. Booster injections of the same type were administered at monthly intervals.

Anti-LRH sera were also produced by adsorbing LRH on polyvinylpyrrolidone followed by emulsification in Freund's complete adjuvant before multiple site injection in rabbits with monthly booster injections as reported by Arimura et al. (1973). Shin and Kraicer (1974) immunized rabbits intradermally with LRH coupled to bovine serum albumin by means of the bis-diazotized benzidine reaction and employing Freund's complete adjuvant. Booster immunizations with the same LRH-conjugate were given every 3 weeks.

A rather different approach was used by Koch and collaborators (1973), who attached p-diazonium phenylacetic acid to synthetic LRH and then coupled the resulting azo derivative to bovine serum albumin by means of the carbodiimide reaction. This resulted in a mixture of azo-tyrosyl and azo-histidyl linkages between LRH and

albumin. Rabbits were immunized by multiple site intradermal injections in Freund's complete adjuvant, supplemented with vaccine against Haemophilus pertussis.

As mentioned above, Frazer & Gunn (1973) managed to couple LRH to bovine serum albumin in a rather simple but obscure manner. They simply mixed 2 mg bovine serum albumin with 2 mg LRH and 75 mg 1-ethyl-3-(3-methyl aminopropyl) carbodiimide and dialysed the mixture overnight against phosphate buffered saline. The product was then used for immunization of two rabbits in Freund's complete adjuvant using multiple site injections. It is not clear how the carbodiimide could affect the conjugation, since LRH has both the C-terminal and the N-terminal blocked and is therefore unable to participate in peptide bond formation. One terminal of the decapeptide is blocked by internal ring formation of the glutamic acid and the other by amidation. The site on the LRH molecule for conjugation is thus not immediately apparent. Possibly, conjugation takes place via the hydroxyl group of the tyrosine or serine residue.

This method was also successful in our hands. After overcoming the initial difficulties with the administration of the conjugate, antibodies suitable for radioimmunoassay of LRH were obtained. The quality of the antibody, especially its specificity, was high enough to keep one rabbit for further antiserum production. Both titre and sensitivity were improved to some extent by the booster injections, but they varied quite independently of each other and no particular pattern was observed.

Most investigators, developing radioimmunoassays for LRH, chose to label with radioiodine by means of the chloramine T method. The method is simple, and as LRH contains a tyrosine residue the reaction is easy to perform. Others, e.g. Miyachi et al. (1973) and Nett & Adams (1977), preferred to use enzymatic methods, but the proof of the superiority of this method has yet to be supplied. In order to improve the quality of the tracer, it is probably better to use a purification step after labeling. Sarda, Barnes & Nair, (1980) could show improved purity, specific activity and immunological behaviour after purifying the labeled hormone by means of batch ion-exchange resin chromatography. In our studies we did not purify beyond the molecular sieve step, as our quality control data indicated that the assay was performing well.

Many different techniques have been used to separate the reaction products from each other after the oxidative iodination. Most investigators used, as we did, Sephadex G-25. Our observation that radioiodinated LRH is retained on Sephadex has been confirmed by other investigators. Nett et al. (1973), who also used Chloramine T radiodination and Sephadex G-25 separation, found two peaks of

radioactivity. The first elution peak could be identified as unreacted iodine and the second as radioiodinated LRH.

Kerdelhue et al. (1973a and 1973b), who also purified the reaction products of a Chloramine T reaction on Sephadex G-25, observed like we did that unlabeled LRH elutes before the radioiodinated LRH. The in vitro biological activity of their labeled preparation comprised 30 - 50% of that of the original preparation. Jeffcoate et al. (1974) showed that the first peak eluting from Sephadex G-25 contains,in addition to unreacted iodine, LRH with diminished immunoreactivity and with enhanced capability of non-specific binding to serum components. This probably represents damaged peptide.

Hichins et al. (1974), when employing another molecular sieve Biogel P2 concluded that the first peak emerging from the column contained the radioiodinated LRH. However, studies by others (Sarda, Barnes & Nair, 1980) indicated that also on this type of column the free iodine and damaged LRH elute before the good quality tracer. By comparing the separation of the reaction mixture on different Sephadex columns, Sorrentino & Sundberg (1975) could show that the best separation of iodinated LRH from the other reaction products can be obtained on G-25 Sephadex. The mechanism responsible for the retention of iodinated LRH on Sephadex G-25 has not been identified. It is presumably due to adsorption induced by the presence of iodine. A similar chromatographical behaviour has been observed for oxytocin (Chard, Kitan & Landon, 1970). This peptide can also be separated from its iodinated form by molecular sieve chromatography on Sephadex G-25.

### 3.4 Summary

A method was found for the preparation of antibody by injecting LRH coupled to bovine serum albumin in rabbits. Radioiodinated LRH could be prepared by the Chloramine T method. A subsequent Sephadex purification step yielded a suitable tracer for the radioimmunoassay of LRH. In the next chapter we shall describe the radioimmunoassay for LRH with this material and the optimal conditions the assay.

# CHAPTER 4

## Radioimmunoassay of the hormone

### 4.1 Introduction

Although all radioimmunoassays are based on the same principle and essentially the same methods are employed to produce the reagents, it is always necessary to establish the optimum incubation conditions for a particular assay. This typically involves the following items. A search for the optimal incubation temperature and reagent concentrations must be made. A separation method for bound and free antigen must be found. A standardized and reproducible method for preparing standard curves must be established. Finally, the assay must be set up in a manner ensuring the highest possible sensitivity and precision. In these studies we used bleedings #3 and #6 from rabbit #14, the one rabbit which produced antiserum with a reasonably high titre. The two bleedings showed similar or identical characteristics in terms of sensitivity, specificity and general performance. In addition we tested two donated anti-LRH sera for specificity and sensitivity.

### 4.2 Optimum incubation temperature and reagent concentrations

The reaction between antigen and antibody is generally temperature dependent, as are all biochemical reactions. Furthermore, the highest sensitivity of the assay will be achieved when incubating at the temperature, at which the affinity constant is maximal. Many, but not all, radioimmunoassays are enthalpy driven reactions. This means that the binding between antibody and antigen will be maximal and also that the radioimmunoassay will be most sensitive at low temperatures. However, the rate of biological reactions is also temperature dependent. All other things being equal, the rate of approach to the equilibrium will be decreased upon lowering the temperature, so that a longer incubation will be required. Association rates between molecules will generally be smaller for large proteins than for small peptides. Factors which play a role are diffusion rates and probability of collision in the proper orientation. This means that we also have to establish the time required to reach equilibrium. These parameters can be established by incubating labeled antigen with antiserum at different temperatures, removing aliquots at various times and then separate bound and free antigen by means of a suitable method.

A personal communication from Dr. S.L. Jeffcoate indicated that relatively short incubation times suffice for the radioimmunoassay of LRH. Consequently, we have used short term incubations for these studies. The optimum incubation temperature was determined as follows: 125-I labeled LRH is mixed with appropriately diluted antiserum and kept at 0, 5, 9, 14, 25 and 37°C for 2, 4 and 6 hours, respectively. After each incubation period 5 volumes of cold ethanol are added to precipitate the antibody-bound fraction (see section 4.3.3 and 4.5 for choice of phase-separation method). After centrifugation and removal of the supernatant containing the free antigen, the remaining radioactivity is measured in the precipitate. Figure 4.1 (right) shows that maximal binding under these conditions is obtained when the incubation is carried out between 5 and 10°C. The results also show that binding is much reduced at and above room temperature.

Such reduced binding could be due to the tracer or antibody being temperature sensitive and breaking down at even slightly elevated temperatures. Consequently, we investigated whether the reduced binding was due to damage or some other mechanism. Timed incubations at 5, 23 and 37°C were carried out (figure 4.1, left). After 4 hours incubation at 23 and 37°C samples were removed and placed at 5°C for another two hours (figure 4.1, dotted lines). Since the binding in these tubes reached the same levels as in the samples kept at 5°C all the time, it follows that the lower binding at the higher temperature is not due to damage of the antibody or the 125-I labeled LRH. This has been confirmed by reversing the experiment: preincubation at 5°C to achieve a preliminary binding, then bringing the tubes to 23 or 37°C for 2 hours and a final incubation for 2 hours at 5°C (figure 4.2). Control tubes were kept at the three temperatures for 30 hours.

The results of these experiments show that binding is dependent on the temperature, and that the processes of binding and release of LRH from the antibody are reversible over relatively short periods of time. The results also show that an incubation time of 4 - 6 hours is sufficient to reach near-equilibrium conditions, since there was over the next 24 hours an increase of only 5 - 10 %.

The reaction between LRH and its antibodies can be assumed to follow the law of mass action. This implies with increasing reactant concentrations the reaction will proceed more to completion during a given time. It means that more tracer will be bound to antibody and hence the measurement of bound antigen will be more precise. The effects of the reagent concentrations on the behaviour of the LRH-radioimmunoassay were determined in a simple experiment by adding different amounts of buffer to fixed amounts of antiserum and labeled LRH. The mixture was incubated at 5°C for 5 hours to ensure

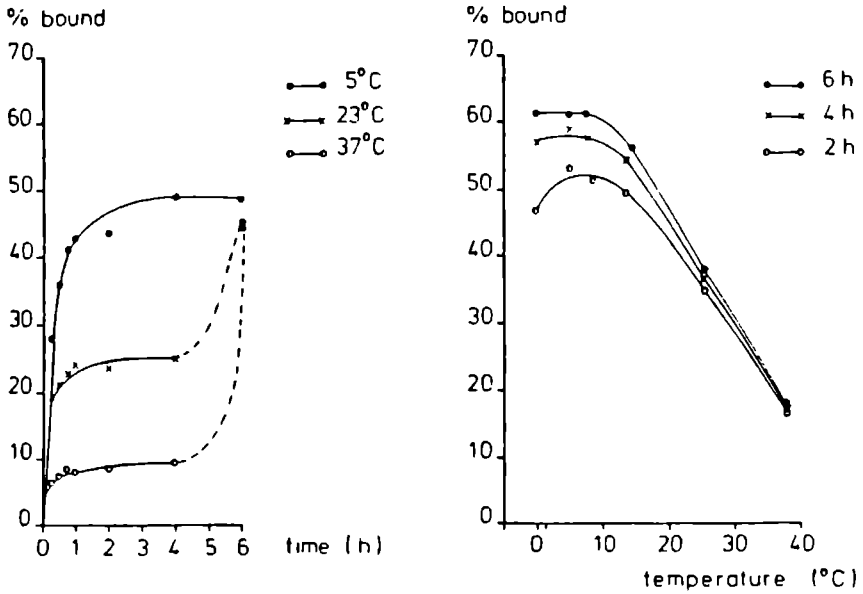


**Figure 4.1: Influence of temperature and incubation time on binding of I-125 labeled LRH to antibody. Bound and free antigen were separated by solvent precipitation.**

near-maximal binding of antigen to antibody (see above). After phase separation by solvent precipitation the radioactivity of the bound fraction was measured. The results are shown in Table IV.1.

**Table IV.1 Dependence of antibody binding on reactant concentration**

| total volume | % bound |
|---|---|
| 0.3 ml | 47.4 |
| 0.4 ml | 43.0 |
| 0.5 ml | 39.7 |
| 0.6 ml | 36.3 |
| 0.7 ml | 34.3 |

As expected, higher binding is achieved at higher reagent concentrations. So the highest possible reagent concentrations should be employed in order to achieve maximal yield in terms of radioactivity.

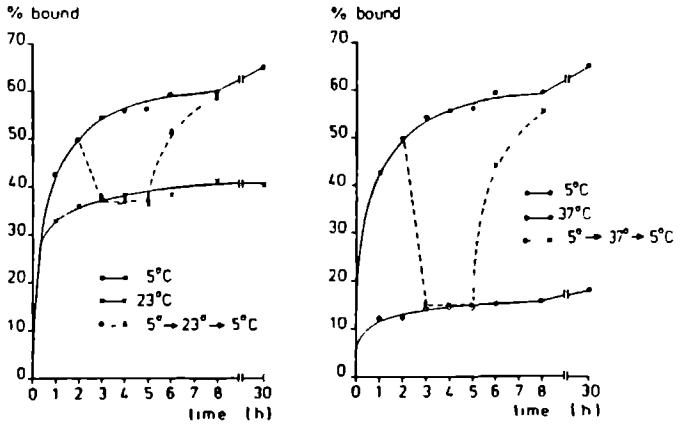


Figure 4.2: Reversibility of I-125 labeled LRH binding to antibody by temperature changes. See text for description of experiment. Phase separation was achieved by solvent precipitation.

Final incubation volumes in the radioimmunoassays are largely established on the basis of very practical considerations, such as availability of precise dispensing equipment and size of disposable reaction vessels. Commonly volumes between 0.5 and 2 ml are used for a typical radioimmunoassay. However, when an extraction procedure is employed in order to, among other things, increase the sensitivity, it is advisable to use the smallest possible volume. Under these conditions a reduction of the final incubation volume from 1.0 ml to 0.1 ml would bring a net increase in sensitivity by a factor of 10. Another reason for using the smallest possible volume is to economize on precious antiserum. On the basis of these considerations, we chose to use 0 1 ml sample or standard, 0.2 ml diluted antiserum and 0.1 ml labeled LRH.

As a general rule, the amount of labeled antigen used in a radioimmunoassay should be approximately equal to the smallest amount of antigen to be measured. Using less tracer allows theoretically for a

more sensitive radioimmunoassay (Rodbard & Lewald, 1970; Rodbard, 1970; Yalow & Berson, 1971). However, in practice there is a limit below which further reduction of the tracer will not produce any improvement in sensitivity (Chard, 1971b; Ekins, 1974). The limiting factor is the increasing level of imprecision as the level of radioactivity decreases. This can be compensated, to a limited extent only, by increasing the counting time. The effect of reducing the tracer concentrations was investigated for the present LRH radioimmunoassay by running standard curves with doubling dilutions of tracer (figure 4.7, see also section 4.4). Radiolabeled LRH, produced as described in section 3.2, was routinely diluted to contain 200,000 cpm/ml, corresponding to 10,000 cpm measured in the zero standard incubation after phase-separation (0.1 ml tracer/tube and approx. 50% tracer bound). This limits the counting error to between 1 and 3 % when counting for 1 minute, which was a desirable time in view of the availability of gamma-counters. The sensitivity of the radioimmunoassay was also acceptable when using tracer prepared in this way.

The concentration of antibody in a radioimmunoassay is often chosen such that approximately 50 % of the tracer is bound to antibody in the absence of competing unlabeled antigen. It has been claimed by Yalow & Berson (1971) that generally the lower the antibody concentration the greater the sensitivity of the radioimmunoassay. However, there is an obvious practical limit here as well, set by the error in the measurement of the bound radioactivity (Ekins, 1974). This is due to an increased fraction of non-specific binding of tracer and any increase in sensitivity is lost to an increase in imprecision. In actual practice binding cannot usually be reduced to much less than 25 %. In our study conditions were chosen such that close to 50 % of the antigen was bound.

## 4.3 Separation of bound and free antigen

### 4.3.1 Introduction

Every radioimmunoassay ends with the measurement of the radioactivity of either free or bound antigen. Mostly this involves a physical separation of the two fractions. In rare instances, a spontaneous precipitation of the antigen-antibody complex will allow phase separation by simple centrifugation. This was accomplished by Walsh et al. 1970 in an assay for Australia antigen. In this case the relatively large size of the antigen and the rather high concentrations of the antigen and antibody made such separation possible. However, in most cases the antibody-antigen complex remains soluble and one of a wide variety of separation methods has to be applied. Table IV.

2 lists the commonly used separation methods.

We chose two of the methods of category 3 for further study: solvent precipitation and double antibody precipitation of the antigen-antibody complex. The double antibody technique has the advantage of an almost universal applicability and the solvent precipitation method is a simple but reliable method utilizing a specific property (difference in molecular mass) of the reagents.

Table IV.2 Methods for the separation of bound and free antigen.

| | |
|---|---|
| 1. Electrophoresis | 3. Antigen-antiboby complex precipitation |
| - starch | - double antibody technique |
| - cellulose acetate | - salting out |
| - polyacrylamide | - solvent precipitation |
| 2. Adsorption of free antigen on a solid phase | - trichloroacetic acid |
| - cellulose | 4. Solid phase antibody |
| - charcoal | - adsorption on tube walls |
| - coated charcoal | - adsorption on disks |
| - talcum powder | - polymerized antibody |
| - QUSO | 5. Gel filtration |
| - ion exchange resins | |

4.3.2 Double antibody precipitation

The double antibody precipitation method is perhaps the most widely used of all phase separation techniques. It was introduced for radioimmunoassay by Utiger, Parker & Daughaday (1962). It depends on the ability of immunoglobulin antibodies to bind to the soluble antigen-antibody complexes and so render it insoluble. The insoluble complex will precipitate and can be centrifuged down, thus effecting a separation of any bound radiolabeled antigen from free labeled antigen present in the incubation mixture. The method is very versatile and it can be used for a large number of radioimmunoassays without changing the incubation conditions.

The anti-immunoglobulins are obtained by immunizing a second animal species with purified gamma globulins derived from the species serving as source for the first antibody. As we were producing anti-LRH in rabbits, the first step was to isolate and partially purify rabbit gamma-globulin. This was done according to the classical method of Cohn (1946) for the separation of plasma proteins by means of cryoprecipitation with various concentrations of ethanol at temperatures below 0°C. Cohn fraction II, which contains gamma-globulins in a very high percentage, could be prepared from rabbit

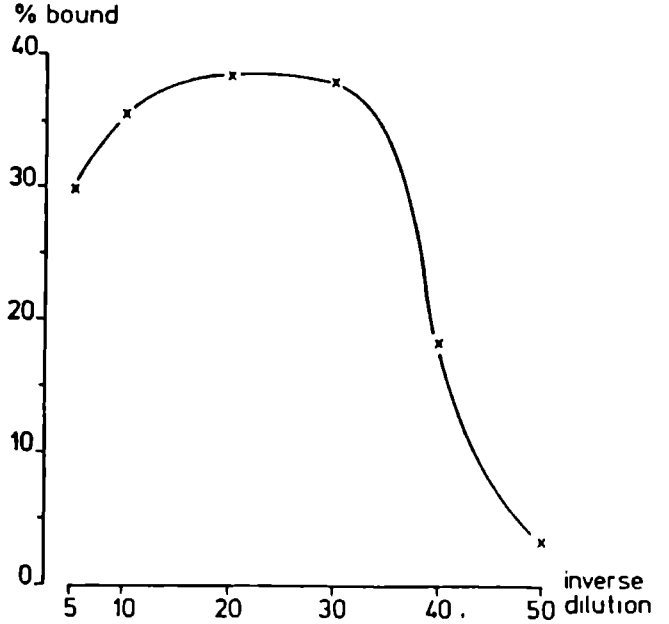


Figure 4.3: Determination of antibody titre for a "second antibody" viz. anti-gamma-globulin. Antiserum to LRH and labeled LRH were incubated under standard assay conditions and this was followed by the addition of serial dilutions of anti-gamma-globulin serum. After 24 hours at 4°C bound and free fractions separated by centrifugation and the radioactivity of the bound fraction was measured.

serum in large quantities and with relative ease. The resulting protein preparation was lyophilized and could be stored at 4°C for very long periods of time. A sheep was injected with the rabbit gamma-globulin fraction in Freunds complete adjuvant and at multiple sites according to Vaitukaitus et al. (1971). The sheep was subjected to jugular phlebotomy at regular intervals. The blood was left to clot at room temperature, rimmed and stored overnight at 4°C for clot retraction. It was then centrifuged to recover the anti-rabbit-gamma-globulin (ARGG) serum. The serum was stored frozen in aliquots at -20°C or as a lyophilized preparation at 4°C.

When setting up a double antibody system for phase separation,

it is necessary to determine the optimal concentration at which the anti-gamma globulin is to be used. The concentration required for maximum precipitation will vary with each antiserum. In the presence of an excess of the first antibody (here: anti-LRH) nearly complete precipitation should occur. On the other hand, as excessive amounts of the second antibody (anti-immunoglobulin) can lead to diminished immunoprecipitation. Hence it is desirable to prepare a dose-response curve for the second antibody. An example of testing a batch of ARGG for use in the LRH radioimmunoassay is shown in figure 4.3. LRH antiserum and radioiodine-labeled LRH in phosphate buffered saline (PBS, see section 3.2) were incubated for 24 hours at 5°C to achieve near-maximal binding of antibody to antigen. This was followed by the addition of sheep ARGG at various dilutions and a further 24 hour incubation at 5°C. At this point 1.5 ml PBS was added and the tubes were centrifuged for 20 minutes at 5000 x g. After removing the supernatant the radioactivity in the precipitate was measured. When the inverse ARGG dilution is plotted against percent bound antigen, a maximum appears at a dilution between 1: 20 and 1:30. Hence, this concentration is preferably used in the LRH radioimmunoassay. Higher concentrations lead to a decreased amount of bound antigen

### 4.3.3 Solvent precipitation of antibody

In the immunoassay of small peptides, as in our case, the separation of bound and free antigen is usually easier than in assays of large proteins. Because of the large differences in molecular mass between the immunoglobulin-antigen complex and the LRH molecule, relatively simple methods of separation can be used. Solvent precipitation is such a technique, where the method depends on the relative ease with which gamma globulins and thus antibody-antigen complexes can be precipitated from aqueous solution by organic solvents. The much smaller LRH molecule can be expected to remain in solution at solvent concentrations precipitating gamma-globulins and their antigen-antibody complexes. This type of separation offers advantages of speed, reproducibility, economy and constancy of reagents.

We have investigated the suitability of this type of phase separation for the LRH radioimmunoassay. Volumes of 100 µl each of radioiodine-labeled LRH, anti-LRH serum or normal rabbit serum and PBS are incubated for 6 hours at 4°C. Then 3 - 10 volumes (0.9 - 3.0 ml) cold ethanol are added, the tubes are centrifuged at 5000 x g for 5 minutes, the supernatant is removed and the radioactivity of the precipitate is measured. The results can be seen in figure 4.4. There is an effective separation of the bound and free LRH fractions over

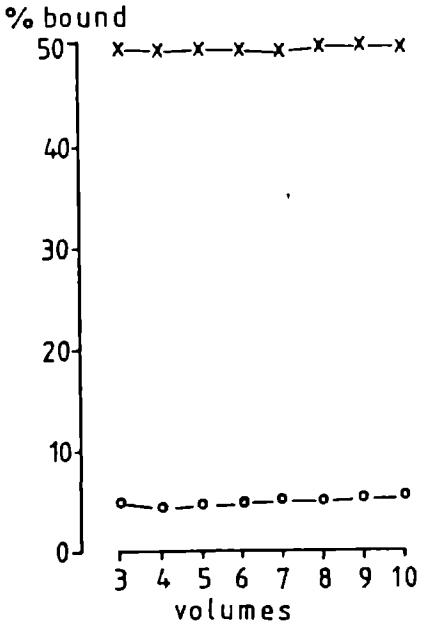


Figure 4.4: Ethanol precipitation separation of bound and free antigen. Labeled LRH was incubated in the presence and absence of anti-LRH serum under standard assay conditions. Ethanol was added, the incubation tubes were centrifuged and the supernatant decanted. The percentage radioactivity in the precipitate without added antiserum is denoted o---o and with anti-LRH serum present is represented by x---x.

the whole range of ethanol additions tested. We conclude that this method also seems to give satisfactory results. Its performance in the LRH assay is described below in section 4.4. We have also investigated whether methanol can be used in place of ethanol. Virtually identical results are obtained, but we have chosen the less toxic ethanol for further assays.

## 4.4 Preparation of standard curves

Many different media can be used in performing radioimmuno-

assays. However, the assay medium must satify the following requirements:

1) The pH and molarity must be close to optimal for the reaction to proceed without interference. In our studies we chose near-physiological molarity (0.14 M NaCl and 0.01 M phosphate) and the pH was kept at 7.0.

2) The assay medium must contain protein in quantities sufficient to prevent loss of reactants on container walls due to non-specific adsorption. Egg albumin in a concentration of 0.1 % provides a cheap and effective means of carrier protection for most applications.

3) In order to provide uniform serum concentrations in all experiments, normal rabbit serum must be added as a carrier to all antiserum-containing solutions at a final concentration of e.g. 1:400.

4) Interference by factors such as complement should be reduced by including 0.05 M EDTA in the incubation mixture.

5) A bacteriostatic agent should be used. Merthiolate at a concentration of 0.01 % provides adequate protection against bacterial growth without interfering with the RIA.

Pure synthetic LRH (Hoechst, Frankfurt) has been used for the preparation of standard curves. Aliquots of 50 µl of a stock solution containing 12.8 ng/ml in the phosphate buffered saline (PBS) are kept at -20°C. Immediately before use serial dilutions are prepared to contain 12.5, 25, 50, 100, 200, 400, 800, 1600, 3200, 6400 and 12800 pg LRH/ml. This provides doubling concentrations (very convenient for plotting standard curves) over the useful range of the LRH radioimmunoassay: 1.25 pg/tube to 1.28 ng/tube when dispensing 100 µl per tube. Standard curves are always prepared in triplicate. Samples were run in duplicate or triplicate, depending on availability. Tubes containing normal rabbit serum instead of anti-LRH serum as well as tubes lacking unlabeled hormone were always included to determine "non-specific binding" and "zero standard value".

There are numerous ways for plotting standard curves obtained from radioimmunoassay data, e.g. the measured radioactivity may be expressed in cpm, % bound, bound/free ratio or free/bound ratio. The constant component (amount of antigen in the standard) may be plotted in an arithmetic or logarithmic manner. A number of logarithmic and logit transformation combinations for both axes have also been employed. Since these methods fail to linearize all types of inhibition curves, there is no single preferable method. For each new radioimmunoassay the best way has to be found by trial and error. A straightforward and readily understood way, which is commonly used, is to plot % bound on the vertical axis against log standard concentration on the horizontal axis. Deviations from normal, such as damaged tracer or antiserum and other binding irregularities, are immediately detectable. It also permits easy comparison of the be-

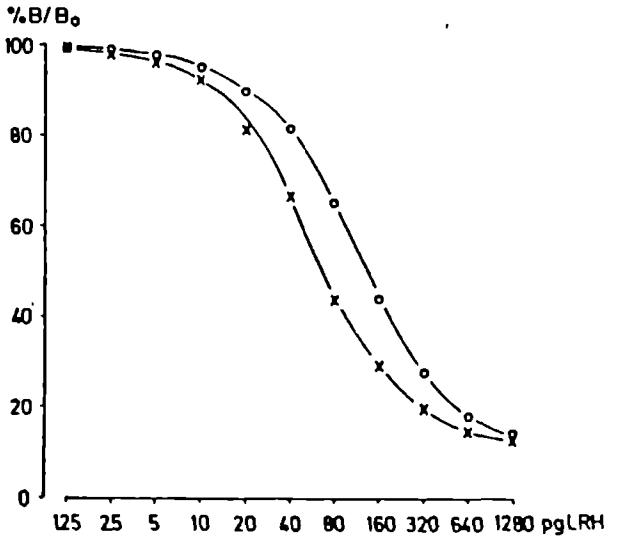


Figure 4.5: Effect of delayed addition of tracer on LRH standard curves. Antiserum and samples were incubated over night under standard assay conditions with (o--o) or without (x--x) labeled LRH present. After 16 hours at 4°C tracer was added to the rest of the incubations. After a further 5-hour incubation bound and free antigen was separated by means of ethanol precipitation.

haviour of individual antisera from assay to assay. If the % bound in the "zero standard" sample is low this suggests deterioration of tracer or antiserum. When this is combined with "non-specific binding" samples and a quality control preparation in every assay, the behavior of the radioimmunoassay sufficiently characterized for daily use.

The method of plotting % bound against log standard has been used throughout this study. In practice we obtained the data from the gamma-counter in the form of triplicate measurements, which were corrected for non-specific binding and averaged. The results were then used for freehand drawing of the standard curves on semi-logarithmic paper, allowing for a best fit with the use of the individual cpm of the triplicates when in doubt. However, for comparisons of different standard curves, as frequently shown in this study, we re-calculate the results. After the usual quality control procedure, we converted to % bound in terms of the cpm of the "zero- standard". This permits a comparison between different antisera or between assay schedules, but it can only be done on pre-screened data as this method of plotting may conceal assay problems.

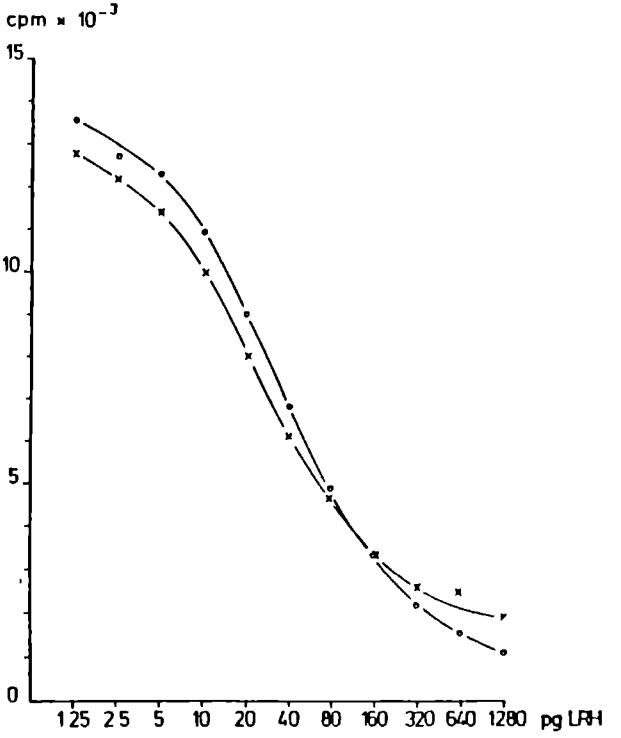


Figure 4.6: Comparison between solvent precipitation and double antibody phase-separation in the LRH radioimmunoassay. Antiserum and tracer were incubated under standard assay conditions over night after which the bound and free fractions were separated by the addition of either ethanol (x---x) or ARGG (o---o).

The order in which reagents are added in a radioimmunoassay is usually: sample - tracer - antiserum. This means that both labeled and unlabeled antigen have equal access to the binding sites. However, preincubation of unlabeled antigen with antiserum, i.e. delayed addition of tracer, increases the sensitivity of the radioimmunoassay (Samols & Bilkus, 1963). This effect is not always found, and thus its applicability to a given system must be established by trial and error. Figure 4.5 shows this effect in the LRH-radioimmunoassay. Standards were incubated with antiserum at 5°C for 16 - 20 hours, followed by the addition of tracer and a further incubation for 4 - 6 hours at 5°C. Separation of bound and free antigen was carried out by means

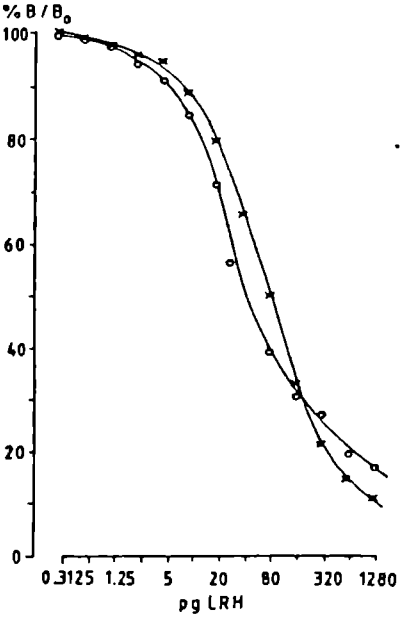


Figure 4.7: Influence of reagent concentrations on the standard curves in LRH-radioimmunoassay. Antiserum and tracer incubated under standard assay conditions but with varying reagent concentrations (see text and table IV.3). Phase separation was done with the solvent precipitation technique. x---x high concentrations, o---o lowest concentrations.

of solvent precipitation. The radioactivity of the bound fraction was measured in a gammacounter. We can see that in this radioimmunoassay preincubation does seem to lead to a more sensitive assay, as suggested by the steeper slope of the inhibition curve is steeper. Whether the difference is really significant and always present was not investigated. However, this method of incubation offers a convenient incubation schedule (overnight and the morning of the following day) and was, therefore used for all subsequent radioimmunoassays in this study.

The method of phase separation may have an influence on the

sensitivity of the radioimmunoassay. Figure 4.6 shows standard curves with the same standard dilutions, the same antiserum solutions and the same 125-I labeled LRH preparation, but using two different separation methods. The inhibition curves are very similar although the double-antibody method yields a slightly steeper slope and could be said to give a slightly higher assay sensitivity.

As pointed out before the sensitivity of a radioimmunoassay can theoretically be improved by employing less concentrated tracer and antibody solutions. This was investigated by running standard curves with different dilutions of the same tracer and antiserum preparations. In order to maintain comparability between the results, reagent solutions were prepared so that the percentage bound antigen remained between approximately 45 % and 55 %. The dilutions and resulting % bound values are presented in table IV.3.

Table IV.3 Effects of tracer and antibody concentrations on % bound to antibody

| cpm / tube | antiserum dilution | % bound |
|---|---|---|
| 10,000 | 1 : 900 | 47.4 |
| 5,000 | 1 : 1050 | 52.7 |
| 2,500 | 1 : 1200 | 56.0 |
| 1,250 | 1 : 1350 | 56.7 |
| 1,250 | 1 : 1500 | 51.3 |

In figure 4.7 the standard curves obtained with the highest and lowest concentrations are shown. A slightly higher sensitivity, as indicated by the slope of the inhibition curve, is found with the lower reagent concentrations. However, this requires a 10-fold longer counting time. Considering the marginal improvement that could be achieved in this way, it was decided to employ conditions as before (10,000 cpm and a 1:900 dilution for this antiserum).

Figure 4.8 shows composite standard curves for three different antisera, two of which were obtained from other investigators:

AS1 - our own production, bleeding # 3

AS2 - antiserum supplied by Dr. S.L. Jeffcoate, London, England

AS3 - antiserum supplied by Dr. G.D. Niswender, Ft Collins, Colorado, USA

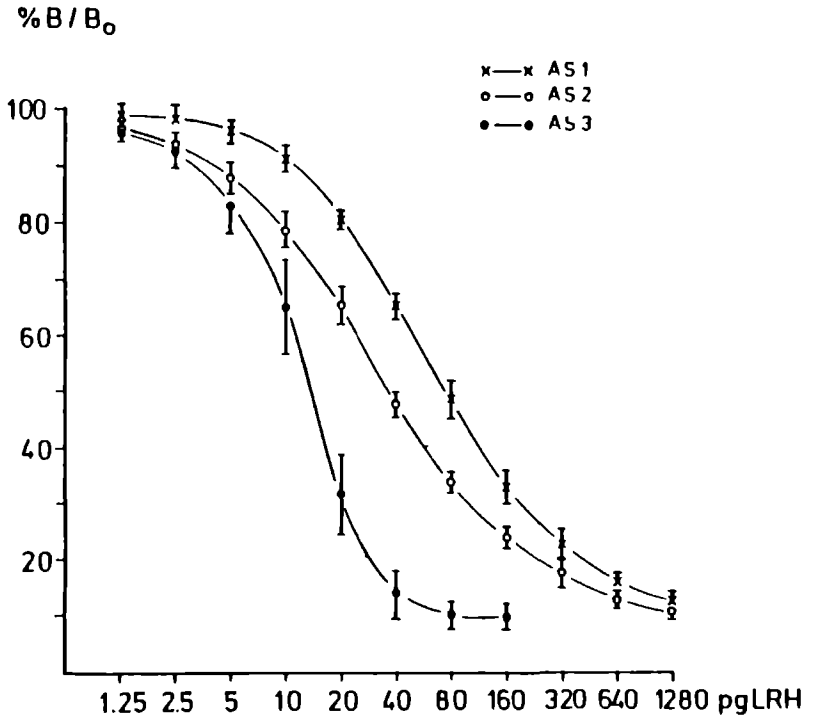


**Figure 4.8: Comparison of three different anti-LRH sera. The points and vertical bars represent mean and standard deviations for six assays using six different tracer preparations. The results are expressed as % bound in terms of the "zero-standard".**

The points and vertical bars represent averages and standard deviations for six assays using six different preparations of 125-I labeled LRH. The sensitivity of a radioimmunoassay is often defined in terms of the variability of the binding to antibody in the absence of standard or sample antigen. Furthermore a value of 2 standard deviations is often used as the limit of statistical significance. Combining the two criteria we can say that the value read from a standard curve that is 2 standard deviations below the amount binding at zero standard provide an estimate of the sensitivity of the assay. For these three antisera we found a sensitivity of 5.7 pg/tube for AS1, 1.9 pg/tube for AS2 and 1.7 pg/tube for AS3, respectively.

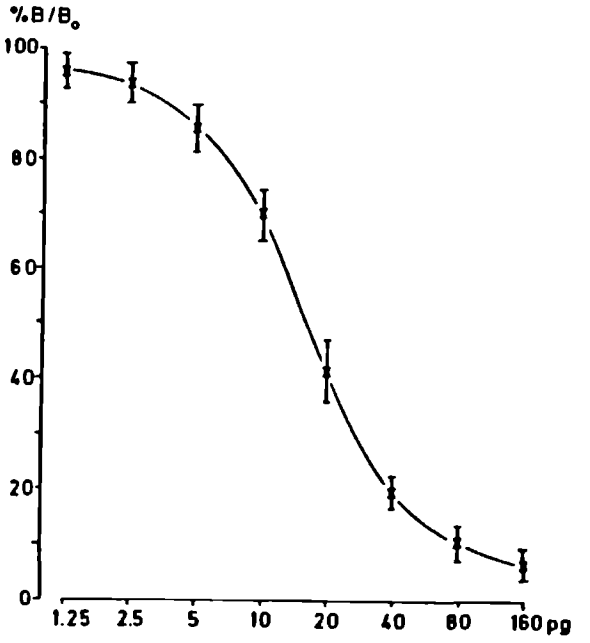


Figure 4.9: Comparison of twenty LRH radioimmunoassays. The points and vertical bars represent means and standard deviation for the standard curve. The results are expressed as % bound in terms of the "zero-standard".

The precision of a radioimmunoassay is evaluated either as the within-assay variance determined by multiple measurements of the same sample in a given assay, or by the between-assay variance determined by multiple measurements in several assays. For the LRH-radioimmunoassay we did this by analyzing the results of 20 assays using the same standard LRH preparation, the same antiserum (bleeding # 6) and different I-125 labeled LRH preparations over an extended period of time. The standards were made up by serial dilutions in the assay buffer, dispensed in assay tubes and kept frozen at -20°C until used. The results from the standard curves were converted into % bound of the "zero-standard" (%B/Bo) and then re-calculated as average and the coefficient of variation, i.e. the standard deviation in % of the average (figure 4.9). The within-assay variation for all the standard points was on average 4.7 % for the 20 assays. When comparing the % B/Bo for the individual standard points between assays we see an average variation (cv) of 7.7 %. Both values are quite acceptable, indicating that our radioimmuno-assay is reliable and reproducible.

4.5 Discussion

The LRH radioimmunoassay presented in this thesis has its optimal incubation temperature at approximately 5°C. Preliminary experiments showed that for the LRH radioimmunoassay the time required to reach equilibrium is relatively short compared to that for larger molecules such as luteinizing hormone. For the latter compound days are needed, whereas binding of LRH. to its antibody is complete after 6 hours of incubation. This means that incubation times of 4 - 6 hours are sufficient for our LRH radioimmunoassay. The reversibility of the binding at higher temperatures indicates decreased binding at higher temperatures is due to kinetics rather than to denaturation of the reactants. These observations have the following practical consequences for the design of the LRH radioimmunoassay:

1) The incubation time in the LRH radioimmunoassay need not exceed 6 hours.

2) Normal refrigerator temperatures provide optimum binding conditions.

3) Temperature during the assay should never exceed 10°C

Other investigators, such as Jonas et al. (1975) used longer incubation times (24 hours), while some investigators employed only 2 hours (Jeffcoate et al. 1972 and 1974a). It seems that incubation time is either not very critical or depends on the individual antisera.

Of the two methods for phase-separation studied by us is the solvent precipitation technique clearly the better choice because of its simplicity and speed and its satisfactory performance. The more elaborate double antibody technique provides a slightly more sensitive radioimmunoassay, but the difference hardly justifies employing this more elaborate and expensive method. In addition, the solvent precipitation method is easier to perform inasmuch as no precision pipetting is required. Consequently, we consider the solvent precipitation method to be the method of choice and have used it in all subsequent experiments. The major disadvantage of precipitation techniques, the relatively high non-specific binding, did not seem to play a role in our assay. These techniques usually yield blanks with values between 5 and 15 % (Chard, 1980), smaller molecules giving lower blanks. In our case 5 % was the normal value, which at a "zero standard" binding of 50 % can be considered as quite acceptable.

Jeffcoate et al. (1972 and 1974a) have also used the method of solvent precipitation. Several other methods have been used for phase-separation in LRH-radioimmunoassays. Dextran-coated charcoal was used by Arimura et al. (1973) and others, while Nett et al.

(1973) and others employed double antibody separation. All these methods work satisfactorily, and thus the choice of method can apparently be made on the basis of personal preference, locally available equipment and other rather pragmatic reasons.

Due to the nature of the LRH producing system and its method of delivering the releasing hormone to the site of action, i.e. secretion into the portal system, we can expect only minute amounts in the general circulation. A lower detection limit in the order of a few pg/ml seems to be a requirement (see also section 6.7). Actually we observe this level of sensitivity in the routine assay. When applying the rather stringent sensitivity definitions described in section 4.4 (figure 4.8), we see statistically significant results at the standard dose levels of 1.7 - 5.7 pg/tube. This sensitivity together with an efficient extraction method (see section 6.3) could be expected to yield physiologically meaningful results in biological fluids. The variability of the assay is at a level where we can hope to be able to make reliable measurements at these concentrations.

## 4.6 Summary

In this chapter we have established optimal conditions for the performance of the LRH-radioimmunoassay, viz. incubation time (4-6 hours), incubation temperature (5°C), concentration of antiserum and radiolabeled LRH. Two different methods for the separation of bound and free antigen have been examined, the double antibody technique and solvent precipitation of the antibody. The latter technique has been chosen for routine use. The preparation of standard curves has been discussed in detail. The type of incubation schedule with the best performance was selected. Three different antisera were compared and the variability of the assay method was established. In the next chapter we shall describe the determination of the immunological and biological specificity of the LRH radioimmunoassay.

# CHAPTER 5

## Specificity of the radioimmunoassay

### 5.1 Immunological specificity

#### 5.1.1 Introduction

One of the main advantages of radioimmunoassays is their potentially very high specificity. Whether this potential is achieved in a given assay depends to a large degree on the nature and quality of the antibodies used. Consequently, the most important single factor in establishing a successful radioimmunoassay is the production of a suitable antiserum. It is often stated that production of antisera with high avidity and specificity is an art rather than a science and that success is determined largely by chance. Be that as it may, it is of paramount importance to assess the specificity of the antisera preparations in order to select the most suitable one. This assessment should also permit the determination of the limitations of the radioimmunoassay in terms of possible interference by other substances.

#### 5.1.2 Definitions and principles

An antigen is a substance that induces antibody formation (ANTIbody GENerator) when introduced parenteraly into an animal (or man for that matter). The term antigen is also used in radioimmunoassay terminology to mean the substance present in a body fluid that we wish to measure. This rather ambiguous terminology has developed over the years and is difficult to avoid. In order to identify the substance used for antibody induction which is frequently another molecular entity than the "antigen", the term immunogen was coined. The immunogen is the substance actually entering the antibody-producing animal. In the case of a small molecule, such as LRH, that must be coupled to a large carrier to become immunogenic, the substance is referred to as a hapten. Injection of the hapten by itself is not inducing antibody formation, but it is capable of reacting with the antibodies induced by the injection of the antigen produced by its coupling to a carrier.

The part of a molecule that is actually combining with the antibody is known as the antigenic determinant, which comprises in the case of a peptides a few amino acids. These amino acids have a

unique three-dimensional arrangement. A change of one amino acid e.g. by addition of a methyl group, or replacement by its stereo-isomer, may impair its binding to the antibody (Berson & Yalow, 1961 and 1966). The area of the hapten that is farthest away from the point where it is attached to its carrier, is often seen to be the antigenic determinant (Parker et al. 1976; Crumpton 1974). Consequently, different conjugation methods may lead to quite considerable differences in specificity.

The part of a molecule that is recognized by an antibody may also be influenced by the type of adjuvant or vehicle that is used when immunizing the animal. For example, an ACTH preparation in gelatin yielded antibodies directed against the C-terminal part of ACTH (Landon, Friedman & Greenwood, 1967), while a zinc complex of ACTH produced antibodies directed against the N-terminal end (Fleischer et al. 1967).

The specificity of an antiserum will depend on the structure of the antibodies involved. Not all antibodies will have the same structure and the serum obtained from an immunized animal may show considerable heterogeneity in its antibody population. Antibodies produced in response to a given antigen may, as a consequence, show different avidities to the antigen, i.e. the strength of the bond between the molecules varies. This explains in part so called cross-reactivity, this means that antibodies with a very low avidity may react with some other molecule. On the other hand, cross-reactivity may result from the inability of an antibody to distinguish between different molecules due to similarities in their structure.

### 5.1.3 Analysis of cross-reactivity

The immunological specificity of antisera can be assessed by running standard curves with a variety of related labeled compounds. The binding characteristics and, more specifically, the displacement from the antibody by the standard substance, can be used to calculate cross-reactivity. A simpler and better method is to run, in the same radioimmunoassay, inhibition curves with as many structurally related materials as possible. Analogues with varying degrees substitution in their amino acid sequence should be employed to obtain a picture of the cross-reactivity. The percentage cross-reactivity is defined as the dose of standard antigen required to displace 50 % of the radiolabeled antigen from the antibody, divided by the dose of analogue required to cause the same 50 % displacement, multiplied by 100 (Abraham, 1974).

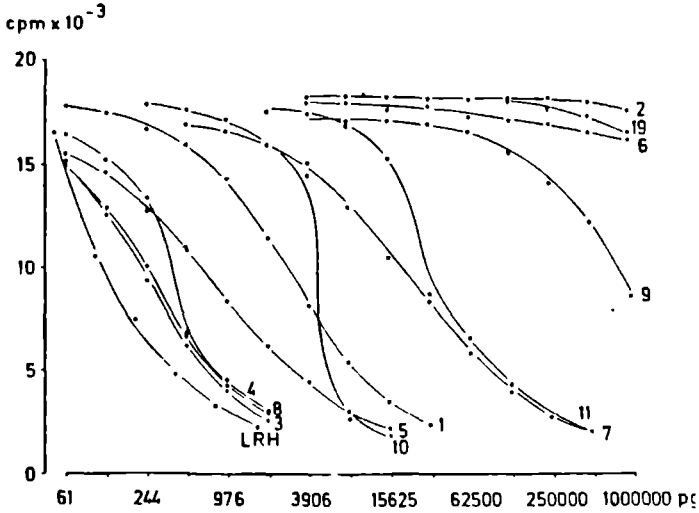


Figure 5.1: Inhibition curves obtained with antiserum AS1 and twelve LRH-analogues. fixed amounts of antiserum and tracer were incubated with varying amounts of the analogues under standard assay conditions. Phase separation was achieved by means of ethanol precipitation. Numbers refer to table VI.1.

In order to establish the immunological specificity of the LRH radioimmunoassay, antisera were incubated with the same series of LRH-analogues as described in the preceding paragraph. These analogues included C-terminal as well as N-terminal fragments and decapeptides with one amino acid of the LRH molecule substituted, kindly supplied by Dr. J. Sandow of Hoechst, Frankfurt. The ability of the analogues to displace 125-I labeled LRH from the antibody was estimated by incubating them with labeled LRH and antiserum as in a radioimmunoassay (described in detail below in section 5.2.2.). Serial dilutions ranging from 1 pg to 1 µg were prepared in the standard assay buffer (PBS with 1 % egg albumin) and were used as unknowns

in the radioimmunoassay. In order to give an impression of the type of inhibition curves that were obtained, the results for one of the tested antisera (AS1) are depicted in figure 5.1. It can be seen that the analogues are giving rise to a wide variety of displacement curves, indicating cross-reactions varying from very close (#3 and #8) to almost non-existent (#2, #19 and #6), parallel and non-parallel.

The results of the specificity studies with the three antisera compared in section 4.4 (in terms of sensitivity and inhibition curves with synthetic LRH) are summarized in Table V.1.

Table V.1 Crossreactivity of three antisera to LRH. Results were obtained by incubating fixed amounts of antiserum and tracer with varying amounts of the displayed LRH-analogues.

| nr. | structure | AS1 | AS2 | AS3 |
|---|---|---|---|---|
| 0 | pGluHisTrpSerTyrGlyLeuArgProGlyNH2 | 100 | 100 | 100 |
| 1 | HisTrpSerTyrGlyLeuArgProGlyNH2 | 3.7 | 54.4 | 3.8 |
| 19 | TrpSerTyrGlyLeuArgProGlyNH2 | n.d. | 74.7 | n.d. |
| 18 | SerTyrGlyLeuArgProGlyNH2 | n.d. | 51.1 | n.d. |
| 2 | TyrGlyLeuArgProGlyNH2 | n.d. | 48.6 | n.d. |
| 17 | ArgProGlyNH2 | n.d. | 0.2 | n.d. |
| 12 | ZSerTyrGlyLeu | n.d. | n.d. | n.d. |
| 13 | pGluHisTrpSerTyrGly | n.d. | n.d. | n.d. |
| 14 | pGluHisTrp | n.d. | n.d. | n.d. |
| 15 | pGluHis | n.d. | n.d. | n.d. |
| 6 | pGluHisTrpSerTyrGlyLeuArgProETHYL | n.d. | 0.02 | n.d. |
| 9 | pGluHisTrpSerTyrGlyLeuArgProPICOL | 0.03 | 0.03 | n.d. |
| 11 | ZSerHisTrpSerTyrGlyLeuArgProGlyNH2 | 0.8 | 55.7 | 0.7 |
| 5 | pGluHisTrpAlaTyrGlyLeuArgProGlyNH2 | 14.0 | 97.9 | 71.0 |
| 3 | pGluHisTrpSerTyrGlyLeuLeuProGlyNH2 | 54.6 | 0.11 | 69.0 |
| 4 | pGluHisTrpSerTyrGlyLeuOrnProGlyNH2 | 58.5 | 0.12 | 53.3 |
| 10 | pGluHisTrpSerTyrGlyLeuhArProGlyNH2 | 56.5 | 0.12 | 50.7 |

n.d.= not detectable, Z is a protective group used in peptide synthesis, ETHYL = ethyl amide, PICOL = picolyl amide, hAr = homo arginine.

Antiserum AS1, (our own antiserum) and antiserum AS3 (supplied by Dr. Niswender) are specific for both the C-terminal and the N-terminal end of the decapeptide, whereas aniserum AS2 (supplied by Dr. Jeffcoate) seems to be specific for the C-terminal only. This can be concluded from the fact that removal of the N-terminal pyroglutamic acid (#1) reduces cross-reaction of antisera AS1 and AS3 to

less than 4%, whilst this reduces the cross-reaction of antiserum AS2 only to 54%. Furthermore, it appears that after replacement of the C-terminal glycinamide with either ethyl- or picolyl-amide (#6 and #9) none of the three antisera are cross-reacting. Antiserum AS2 crossreacts with the C-terminal hexapeptide Tyr-Gly-Leu-Arg-Pro-Gly-amide (#2) to almost 50 %, indicating that this antiserum ignores a large part of the N-terminus. On the other hand, antiserum AS2 shows a high degree of dependency on the arginine in position 8. When this amino acid is replaced by leucine (#3), ornithine (#4) or homo-arginine (#10) the cross-reaction is substantially reduced for antiserum AS2 but to a much lower degree with the other two antisera. For antiserum AS1 we see a higher dependency on the alanine residue in position 4 (#5).

We have also investigated whether the high specificity obtained in one of the rabbits changed during the course of immunization. The first six bleedings from this rabbit were checked as described above with one difference. Only des-pGlu-LRH (#1), LRH, and des-Gly-amide-ethyl-amide-LRH (#6) were used. These two peptides lack either the C-terminal or the N-terminal end of the LRH molecule, and thus their degree of cross-reactivity will provide the essential information - the C- terminal and N-terminal specificity of the antisera. The results, shown in Table V.2, indicate that the antisera from this rabbit are consistently of the same high specificity.

Table V.2 Specificity of LRH antisera obtained from sequential bleedings of the same rabbit.

| Bleeding number | % cross-reaction | |
|---|---|---|
| | C-terminal lacking | N-terminal lacking |
| 1 | n.d. | 4.7 |
| 2 | n.d. | 3.9 |
| 3 | n.d. | 4.4 |
| 4 | n.d. | 3.9 |
| 5 | n.d. | 1.5 |
| 6 | n.d. | 2.9 |

n.d. = not detectable

## 5.2 Biological specificity

### 5.2.1 Introduction

It is known that many hormones may be present in the organism in several forms, in addition to the actual biologically active form, e.g. as precursors or metabolic breakdown products. Any of these forms may contain the immunologically recognized structure(s) for a certain antibody. Precursors have been identified for LRH (Millar et al. 1977; Galton, Pattou & Kordon, 1981; Seeburg & Adelman, 1984) and metabolic breakdown products have also been reported (Jeffcoate & Holland, 1975; Marks & Stern, 1974)

The accuracy of a radioimmunoassay depends on the identity or association between the structural components determining the biological activity and the components responsible for the immunological binding. A comparison of measurements based on biological assay principles with those based on radioimmunoassay is therefore an essential part of the validation of a radioimmunoassay. Even though our LRH radioimmunoassay has been shown in section 5.1 to possess excellent immunological specificity we still deemed it necessary to compare with an established bioassay, the Ramirez-McCann in vivo assay for LH-releasing activity.

### 5.2.2 Radioimmunoassay of the hormone

Based on the results of the experiments described in chapter 4 we set up a standard method for the radioimmunoassay of LRH. Sample size for unknowns and standards was set at 100 µl. Standards and unknowns in the cases where extractions were done, were dissolved in PBS-0.1% egg albumin (see section 3.2). The samples, in 10 x 75 mm tubes, were put on ice. Appropriately diluted antiserum (200 µl) was added, and the content of the tubes was mixed on a Whirlmix. The antiserum had been diluted in PBS-0.1% egg albumin containing normal rabbit serum to give a final dilution of 1:400 with EDTA added to yield a final assay concentration of 0.05 M. The mixture was incubated at 4°C over-night, after which 100 µl of 125-I labeled LRH was added in PBS- 0.1% egg albumin. The tubes were kept on ice at all times and never allowed to reach room temperature. After mixing the tubes were incubated for a further 5 - 6 hours at 4°C. The bound and free fractions were separated by the addition of 5 volumes cold ethanol followed by centrifugation. After decanting the supernatant, the radioactivity of the precipitate was measured.

5.2.3 Bioassay of the hormone

The first attempts to assay LH-releasing activity were made by measuring the ovarian depletion of ascorbic acid in immature rats, pretreated with gonadotrophins. Since this test animal is highly sensitive to LH, the only way to distinguish between LH and LRH in this system was to treat the samples by heat, as LH is heat labile and LRH is not.

A much better method was later developed by Ramirez & McCann (1963). They took advantage of the fact that ovariectomy will lead to removal of ovarian steroid feed-back. This results in an open-loop system which is highly sensitive to LRH stimulation. However, this treatment has the disadvantage that the pituitary is already stimulated to a very high degree by endogenous LRH. This leads to very high LH levels in the blood, making it difficult to detect any increases. Furthermore, the high stimulation level may result in a decreased capacity to respond to any further stimulation. In experiments designed to block LH releasing activity Ramirez and McCann pretreated the ovariectomized rats with large doses of oestrogen and progesterone. To their surprise they found that the rats had an increased sensitivity to LRH rather than a block. Apparently this treatment lowers LH blood levels, while at the same time maintaining the high sensitivity to LRH stimulation presumably due to the highly elevated pituitary LH stores. This method became the standard in vivo bioassay for LRH activity.

In the original LRH bioassay, another bioassay, the ovarian ascorbic acid depletion method was used to determine changes in plasma LH levels after injection of the test substances. The advent of radioimmunoassays for rat LH led to improvements in the sensitivity of the bioassay and to its simplification. In these experiments we have employed a radioimmunoassay for plasma LH developed by Niswender et al. (1968). It uses anti-ovine LH serum and radiolabeled ovine LH and a rat standard preparation. The specificity of this assay was established by measuring LH content of several pituitary preparations with widely varying LH/FSH and LH/TSH ratios. The observed behaviour of serum LH levels was consistent with known physiological processes.

The LH antiserum used in these studies was GDN#15, supplied by Dr. Niswender. Ovine LH for radioiodination by the method of Greenwood et al. (1963) and the standard preparation (rat LH-RP1) were supplied by the National Institute of Arthritis and Metabolic Diseases, NIAMD, Bethesda, MD, USA. The assay was carried out by placing samples or standards in a total volume of 0.5 ml phosphate buffered saline (PBS, see section 3.2) containing 1 % egg albumin. This was followed by addition of 0.2 ml appropriately diluted anti-

ovine LH serum. The antiserum was diluted 1:20,000 in a buffer consisting of normal rabbit serum diluted 1 : 400 in PBS-1% albumin. The content of the tubes was then mixed and incubated at 4°C for 24 hours, whereupon 0.1 ml 125-I labeled ovine LH was added. The radiolabeled LH had previously been purified on a Sephadex G-50 column and was then diluted with PBS-0.1 % egg albumin to contain approximately 10,000 cpm/incubation tube. After a further 24 hours at 4°C 0.2 ml anti-rabbit-gamma-globulin (see section 4.3) was added to each tube to precipitate the antigen-antibody complex. After 72 hours at 4°C bound and free antigen could be separated by adding 3 ml cold PBS and centrifuging at 1000 x g for 30 minutes. The supernatant was decanted and the radioactivity of the precipitate was measured in a gamma counter.

The performance of the LH-radioimmunoassay was checked by evaluating between-assay-variation as well as within-assay-variation. This was done by including a quality control preparation made from pooled rat serum from normal rats sacrified for other experiments. The preparation was measured 3 to 5 times in every radioimmunoassay and the coefficient of variation (cv) calculated from the results. From 12 radioimmunoassays we obtained an average within-assay variation of 3.4% with a range of 0.82 to 11.61%. The between-assay variation, calculated from the same results, amounted to 8.4 % (coefficient of variation).

### 5.2.4 Comparison of the immunoassay and the bioassay

For the standard Ramirez-McCann bioassay for LH-releasing activity female Sprague-Dawley rats, weighing 120 - 150 grams, were bilaterally ovariectomized between 1 and 3 months prior to their use in these studies. The rats were prepared for the bioassay by subcutaneous injection of 50µg oestradiol benzoate (Progynon B oleosum dissolved in sesame oil) and 25 mg progesterone (Progesterone "Vitis" dissolved in sesame oil). After three days the rats were placed under ether anasthesia and the jugular vein was exposed. A 1-ml blood sample was taken from each rat, followed by the injection of a LRH solution into the jugular vein through the sampling syringe. The blood was immediately centrifuged and the plasma was snapfrozen. It was kept at -30°C until radioimmunoassay of the LH content. Exactly 10 minutes after the injection of LRH a second blood sample was taken in the same manner. The LRH doses tested in this experiment varied between 1 ng and 10 ng, which according to earlier experiments covers the range of a linear semilogarithmic plot of the bioassay. The releasing hormone preparations were prepared by serial dilution in the normal phosphate buffered saline containing 1 % egg albumin to minimize losses due to adsorption on tube walls and other

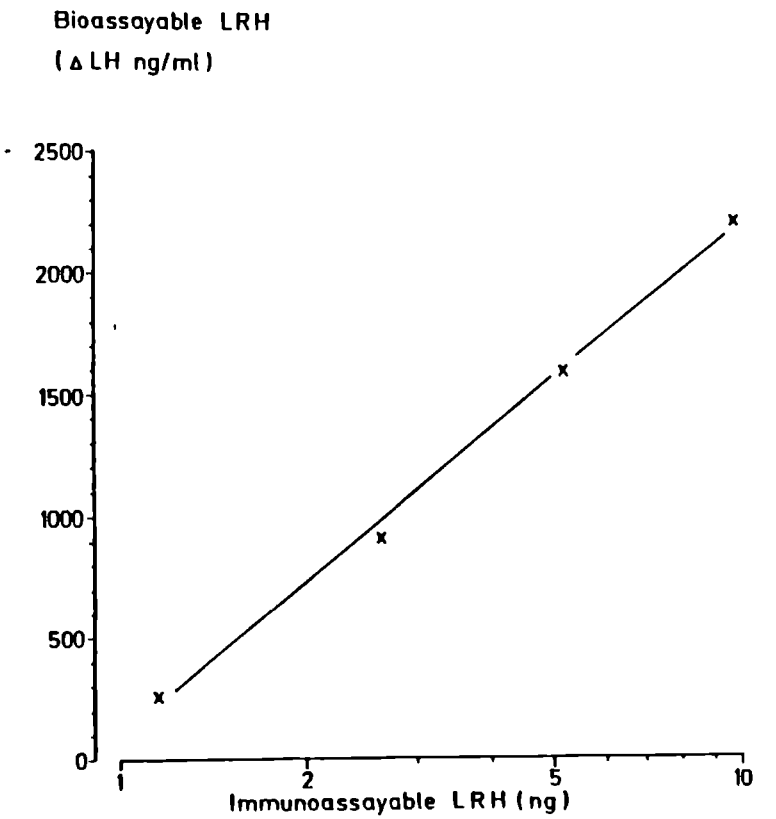


Figure 5.2: Comparison between our radioimmunoassay and a standard bioassay (Ramirez-McCann assay) for LRH. Results of the bioassay are expressed as the increase in plasma LH (rat LH RP#1) in response to the LRH administration.

materials. Five rats were used for each dose of LRH and the average results calculated from triplicate determinations in the LH radio- immunoassay. The same preparations were also subjected to LRH radioimmunoassay as described in section 5.2.2.

The results of the two assays are displayed in figure 5.3. Bioassay results are plotted on the vertical axis as the change in radioimmunoassayable plasma LH caused by the releasing hormone injection. On the horizontal axis the results of the direct LRH radioimmunoassay are shown in logarithmic fashion. The linear relationship between the two parameters indicates that there is an excellent agreement between the two assay methods under these conditions.

## 5.3 Discussion

Antisera AS1 and AS3 were found to be specific for both ends of the LRH molecule. The studies of Nett et al (1973) and Jonas et al (1975) using antiserum AS3 and a later bleeding from the same animal, in analogy to our studies, showed limited or non-detectable cross-reaction with LRH analogues having either the C-terminal or the N-terminal amino acid removed or substituted. Antiserum AS2 is specific for the C-terminal end only, as has previously been reported by Jeffcoate et al. (1973). Their studies showed complete cross-reaction from the 3 - 10 octapeptide and an almost complete lack of cross-reactivity from the 1 - 9 nonapeptide. The specificity difference between AS2 and AS1 is not easy to explain, since the immunization procedures were not greatly different and the same coupling technique had been used for producing the LRH-BSA conjugate. The mechanism of this conjugation method is not clear, as the decapeptide LRH has both terminals blocked for peptide bond formation. The N-terminus has an internal ring structure and the C-terminus has an amide group attached. As mentioned earlier, the conjugation site is important for the specificity of the produced antiserum. It is conceivable that the conjugation method is not very specific and may thus yield different results in different laboratories, giving rise to conjugation at different sites of the LRH molecule and leading to different specificites.

The fact that antisera AS1 and AS3 are specific for both ends of the LRH molecule should make them immunologically highly specific, since any LRH metabolite is likely to have at least one terminal amino acid missing. In this respect, these antisera should be superior to antiserum AS2, which is specific for the C-terminal end only.

An immunogen may contain several antigenic determinants, some strong, some weak. The initial response in the course of immunizations will likely be directed towards the strong determinants. However, as the immunization proceeds over a period of perhaps many months a diversity of antibodies may develop, as also the weaker antigenic determinants may become involved. This has been observed in the production of antibodies to collagen by Timple et al. (1972). During the early phases of antibody production the antibodies were predominantly directed towards the C-terminal ends of the polypeptide chains of collagen. In a later phase antibodies against the N-terminals appeared and the production of antibodies for the C-terminals diminished. Thus, in the course of an immunization over an extended period of time it is desirable to investigate whether the initial specificity is maintained. In our case, the one rabbit producing suitable antisera generated for several months an antiserum which had an invariably high specificity for both ends of the LRH molecule for several months. Hence, all the bleedings could be used and a large

amount of antiserum could be stockpiled for later use.

Since the antigenic determinant need not correspond to the amino acid sequence responsible for the biological activity of the compound, it is obvious that in this way serious discrepancies can arise between the immunological and biological activities. A radioimmunoassay may thus, in addition to the desired compound, measure biologically inactive fragments, denatured antigen retaining the antigenic determinant, and other compounds containing elements structurally similar to the antigenic determinant.

The bioassay used for comparison with the radioimmunoassay in this study, the Ramirez-McCann assay, is the most sensitive in vivo bioassay for LH releasing activity (McCann, 1983b). According to the originators of the method it can be used to detect approximately 1/10 of a rat stalk median eminence equivalent (Ramirez et al. 1975). This corresponds to about 0.3 - 1 ng LRH depending on the size of the rat (see chapter 7). We found the the same order of magnitude for the detection limit as reported by Ramirez et al. (1975), viz. basal LH levels of 50 - 150 ng/ml (in terms of the NIAMD RP#1) and an increase by 200 - 300 ng/ml after injection of 1.25 ng LRH. The bioassay could possibly be made somewhat more sensitive, but is unlikely to yield a sensitivity below 0.1 ng. This compares rather unfavourably with the approximately 1 - 5 pg lower detection limit of the radioimmunoassay. The excellent agreement between the bioassay and the radioimmunoassay for LRH, also noted by e.g. Ramirez et al. (1975), for a different LRH radioimmunoassay confirms that the immunoassay can indeed be very specific. Under the conditions used by us, it should yield a true measurement of releasing hormone activity provided certain precautions are taken to eliminate plasma interference.

## 5.4 Summary

In this chapter we have investigated the specificity of the LRH-radioimmunoassay. The immunological specificity was established by means of measurement of the ability of LRH-analogues to displace radiolabeled LRH from binding to the antibodies. Of the three antisera checked, two were found to be highly specific in that they required both ends of the LRH molecule for binding, the third was found to be specific for the C-terminal only. It could also be established that the high specificity was maintained during a prolonged immunization period. The biological specificity of the radioimmunoassay was checked after establishing a bioassay for LRH, which in turn required the setting up of a radioimmunoassay for rat LH. We found excellent agreement between the two assay methods. Further

evidence validating the LRH radioimmunoassay is provided by the parallel inhibition curves for hypothalamic extracts and synthetic LRH in chapter 7. In chapter 6, we shall describe the radioimmunological measurement of LRH in plasma.

# CHAPTER 6

## Measurement of the hormone in plasma

### 6.1 Introduction

The assay of LRH in plasma encounters various problems. First, LRH like other hypothalamic releasing hormones is essentially a local hormone, which is primarily secreted into the hypothalamo-pituitary portal circulation. This implies that we can expect only minute concentrations of hypothalamic LRH to be present in the general circulation. As the sample size in a radioimmunoassay is limited, an extraction procedure is needed to increase assay sensitivity sufficiently to permit determination of LRH in plasma samples. A second potential problem of a more general nature is the danger of enzymatic degradation of LRH in biological fluids. A third potential problem is that binding of the releasing hormone to macromolecular components of plasma or serum may occur and affect the assay results. finally, LRH may be secreted, as is LH in an episodic manner and there may be a relatively rapid plasma clearance of LRH.

The first three of these potential or real problems in the assay of LRH in plasma could conceivably be overcome by the use of a suitable extraction procedure. Such a method must fulfill several requirements (Chard et al. 1971b and 1973). The hormone should, of course, be present in a higher concentration after the extraction. The extraction procedure must be rapid and simple, not only for the sake of efficiency, but also to prevent degradation of LRH by proteolytic enzymes. Non-specific inhibitors and proteolytic enzymes must be eliminated. The recovery must be reasonably high and reproducible. The chemical identity of the hormone must be preserved during the extraction, so that its immunological activity will not be affected.

### 6.2 Direct measurements in plasma

Initial attempts to measure LRH in plasma often showed no detectable LRH. In order to investigate whether plasma degradation of LRH plays a role in this phenomenon, we prepared a pool of aged plasma by combining a number of normal samples which had been received for routine gonadotrophin measurements. The plasma pool was left at 4°C for 30 days in order to allow for termination of all potential reactions. LRH was added at four different levels (160, 320, 640 and 1280 pg/ml) and the fortified plasma samples were incubated at three different temperatures (4, 20 and 37°C). Samples were

removed after 4 hours and after 24 hours and they were rapidly frozen. The samples were then thawed and simultaneously analyzed by means of radioimmunoassay with two different antisera, AS1 (our own antiserum, bleeding #3) and AS2 (Dr Jeffcoate's antiserum). Sampling was done in quintuplicate. The results, summarized in Table VI.1, represent averages for four dose-levels of LRH added.

TABLE VI.1 Stability of exogenous LRH in plasma.

| | % recovery with AS1 | | % recovery with AS2 | |
|---|---|---|---|---|
| temperature | 4 hours | 24 hours | 4 hours | 24 hours |
| 5°C | 90 | 57 | 106 | 68 |
| 20°C | 68 | n.d. | 72 | 16 |
| 37°C | 47 | n.d. | 61 | 12 |

n.d.= not detectable

It is obvious that the plasma samples contain proteolytic activity and thus that a countermeasure should be employed. It can also be seen that the antiserum with the less specific antiserum AS2 (which is specific for the C-terminal of the LRH molecule only) gives higher values, probably reflecting the presence of metabolites, i.e. proteolytic fragments.

Another problem associated with the radioimmunological measurement of LRH is also seen in Table VI.1, viz. the 106 % recovery after 4 hours at 5°C as measured by antiserum AS2. We often encountered such effects during the preliminary attempts to measure LRH in plasma, frequently with "recoveries" higher than the above 106% Such non-specific interference by plasma with the binding of LRH to antibody has also been reported by others (Arimura et al. 1973).

An obvious way to circumvent this problem would be to prepare all standard curves in LRH-free plasma, while at the same time taking care to prevent enzymatic degradation. This was achieved by using aged plasma, obtained as above, and preparing the standard as rapidly as possible and keeping all reagents at or below 4°C at all times. The feasibility of this approach was tested by preparing ten standard curves with serial dilutions of synthetic LRH in ten different (aged) plasma pools at ten different occasions. These preparations were then subjected to a normal LRH-radioimmunoassay as described in section 5.2.2. The results, shown in figure 6.1, are represented as average % bound (%B/Bo) with standard deviation for

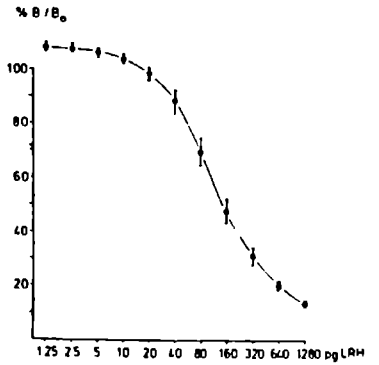


Figure 6.3: Preparation of standard curves in plasma with prior extraction. Averages with SD from 10 assays.

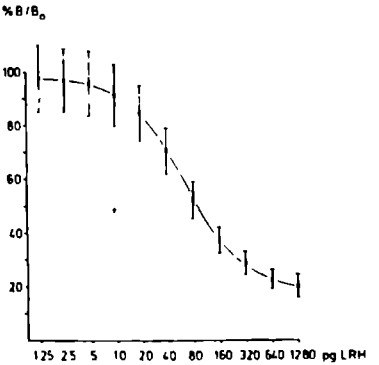


Figure 6.1: Preparation of standard curves in plasma without prior extraction. Averages with SD from 10 assays.

each dose of LRH. The very high variation between the individual standard curves shows that direct measurement of LRH in plasma can lead to completely misleading results. This is presumably due to that plasma from different individuals may contain factors interfering to different degrees and in different manners, i.e. plasma compensation alone is not sufficient. A falsely negative as well as a falsely positive value is equally possible. An overestimate would arise when the plasma interference caused a decrease in binding of labeled LRH to antibody. Similarly, a reduction of apparent LRH levels would result when the plasma caused an increase in binding. Both these effects were seen in the above individual plasma standard curves.

### 6.3 Extraction procedure for LRH plasma samples

In view of the above observations we have included an extraction procedure preceding the measurement of LRH in plasma samples. The procedure consists of adding 10 volumes of cold ethanol to the sample, mixing for 10 seconds on a Whirlmix, centrifuging off the precipitate and removing the supernatant to another test tube.

The sediment was washed to remove any remaining LRH by adding a further 5 volumes ethanol followed by mixing, centrifugation and decanting of the supernatant. The two supernatants were combined and evaporated to dryness under a stream of dry nitrogen in a water bath at 50°C. The dry residue was dissolved in assay buffer (PBS with 1 % egg albumin) and subjected to a standard LRH radioimmunoassay (section 5.2.2).

The extraction method was checked for performance and recovery by preparing a normal series of standards in assay buffer, splitting each sample in two parts and subjecting one of them to the extraction procedure before assay, while using the other part without extraction. The results, shown in figure 6.2, demonstrate an almost complete overlap of the two standard curves, indicating a virtually complete recovery. It also seems that using this procedure there is a neglible loss of LRH due to proteolytic activity. Furthermore, the extraction method allows for a plasma sample of up to 1 ml, as the standard curves are drawn as pg LRH/determination we acchieve a sensitivity of a few pg LRH/ml plasma. This means an approx. ten-fold increase above the straight sampling method.

### 6.4 Measurement of the hormone in plasma extracts

We investigated whether the above extraction procedure could eliminate the spurious results obtained with standard curves prepared in plasma as shown in figure 6.1. Experiments analogous to those in section 6.2 were carried out, but this time the samples were first extracted. Again, ten aged plasma pools were prepared at ten different occasions, LRH was added and serial dilutions with the appropriate LRH concentrations were prepared. Ethanol was added immediately and the extraction procedure carried out as described above in section 6.3. The results, shown in figure 6.3, are again presented as averages with standard deviations. The resulting variations that are observed are now well within the normal range for a proper LRH-radioimmunoassay, indicating that plasma non-specific interference can largely be eliminated by this extraction procedure.

### 6.5 Measurement of the hormone in rat plasma

At a signal from higher brain centres involved in circadian rhythm control the hypothalamo-pituitary system responds with a LH surge, resulting in ovulation. Thus, the highest plasma LH levels in

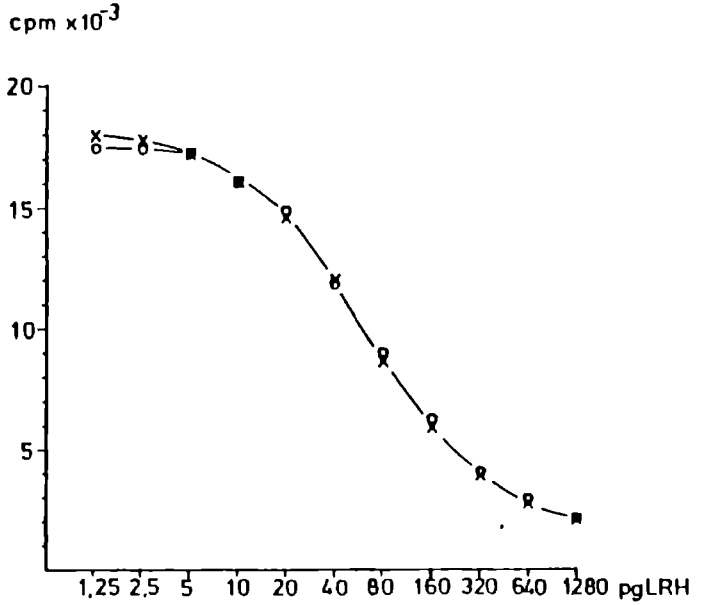


Figure 6.2: Comparison of standard curves for extracted and non-extracted standard mixtures

the rat are found during the pro-oestrous phase of the reproductive cycle. It follows that we have a large probability of finding the highest plasma LRH levels at or around the time of ovulation. However,' there are quite large variations between individual rats in terms of time of ovulation, length of oestrous cycle and maximum LH levels. So we decided that relying on the reproductive cycle might not be the best experimental design and that more standardized conditions would be preferable.

It is well established that the ovulatory surge of LH in the rat is induced by gonadal steroids acting on the preoptic/hypo- thalamic regions of the brain (Everett, 1964; Davidson, 1969; McCann, 1974). Steroids can act at both the pituitary and hypo- thalamic levels to inhibit gonadotrophin release (Bogdanove, 1964; Davidson, 1969; McCann, 1974), but also to sensitize the pituitary gland to releasing hormone action directly (Kalra et al. 1973; Libertun et al. 1974; Debeljuk et al. 1974). The most interesting effect of exogenous steroids in this context is a massive gonadotrophin release some 6 hours after injection of oestrogen-primed, ovariectomized female rats with progesterone (Caligaris et al. 1968 and 1971; Kalra & McCann, 1973). This artificial LH-surge seemed to provide conditions with a

reasonably high probability of finding elevated LRH levels in the general circulation.

Twelve mature female rats, which had been ovariectomized not less than six months earlier, were injected subcutaneously with 5 µg oestradiol benzoate (Progynon B oleosum) in 0.5 ml sesame oil. After two days six of these rats were injected with 1.5 mg progesterone (Progesterone Vitis) in 0.5 ml sesame oil and the other six rats with sesame oil only. A blood sample was collected under ether anesthesia from the jugular vein of each animal at the time of the oestradiol injection and of the progesterone injection. Further samples were collected in the same manner 1 hour and 6 hours after the progesterone treatment. Plasma was prepared by centrifugation of the blood samples in a refrigerated centrifuge and was frozen immediately. Samples were kept at -30°C until performance of the LRH-radioimmunoassay with prior extraction as described in section 6.3. The results are presented in Table VI.2, expressed as pg LRH / ml plasma.

Table VI.2 Plasma immunoreactive LRH levels in rats before and after progesterone injection in oestradiol-primed castrates.

| group | day 0 | day 2, 0h | day 2, 1h | day 2, 6h |
|---|---|---|---|---|
| control | 30+/-6 | 32+/-1 | 17+/-2 | 25+/-2 |
| treated | | 20+/-6 | 28+/-6 | 33+/-8 |

Oestradiol priming took place on day 0, progesterone injection on day 2, 0h. Averages with SEM are given

The results on day 0 were pooled as all rats had been treated in an identical manner up to this point. Although the levels of immunological LRH are above the detection limit of the assay, no statistical differences are observed either in time within the groups or between the treatment groups. We must therefore conclude that no significant increases in immunoassayable plasma LRH in response to the steroid treatment have been detected. This could possibly be due to the sampling technique, the LRH output responsible for the LH release may have been of short duration only.

## 6.6 Continuous recording of the plasma hormone level in man

When several blood samples are collected at short time intervals and estimated for hormone content, one will often find that the hormone levels fluctuate from sample to sample. This phenomenon was first recorded in the secretion of ACTH by Berson & Yalow (1968). Since then similar evidence has accumulated for a number of other hormones such as LH (Dierschke et al. 1970; Yen et al. 1972), FSH (Naftolin et al. 1972), growth hormone (Takahashi et al. 1968), TSH (Vanhaelst et al. 1972). Detailed examination of plasma LH concentrations shows sharp oscillations. The periodicity varies, but it is of the order of one to several hours. It seems that there is no maintenance of a steady "basal" gonadotrophin level, but rather that there are abrupt peaks of episodic pituitary discharge separated by periods without secretion. The magnitude of the pulsatile LH release varies with the endocrine milieu. The highest release is found under conditions of low steroid feed-back, such as after castration (Yamaji et al. 1972) or menopause (Yen & Tsai, 1972). The frequency of the LH secretory bursts is also variable. Yen et al. (1972a) could show differences in frequency over the different stages of the human menstrual cycle.

Consequently, when samples are being collected for hormone determination, intermittent sampling techniques can conceivably give misleading or even false information about secretory patterns. This is particularly true where the sampling interval is greater than the half-life of the hormone being investigated. An obvious solution to this problem is to employ continuous sampling, which will enable the investigator to obtain the mean hormone level for the collection period. The collection time required for a given hormone will depend on the half-life of the hormone. LRH has a relatively fast disappearance of the order of 3 - 4 minutes half-life (Keye et al 1973; Arimura et al. 1974). Hence, in this case a sampling time of the order of a few minutes is required.

This led us to carry out a series of measurements of immunoassayable plasma LRH in patients and volunteers, employing a system for continuous sampling. A jugular vein catheter was inserted via the cubital vein and connected to a peristaltic pump. Blood was continuously withdrawn at a rate to yield approx. 5 ml blood per 5- or 10-minute collection period. The blood samples were collected in heparinized tubes kept in an ice-bath. After sampling was completed, each individual tube was briefly mixed on a Whirlmix and centrifuged in a refrigerated centrifuge to obtain the plasma. Plasma aliquots were frozen for LH determinations as described in chapter 8 (Dahlen et al. 1974). For the LRH determinations 1 ml aliquots were immediately extracted and assayed as described in section 6.3.

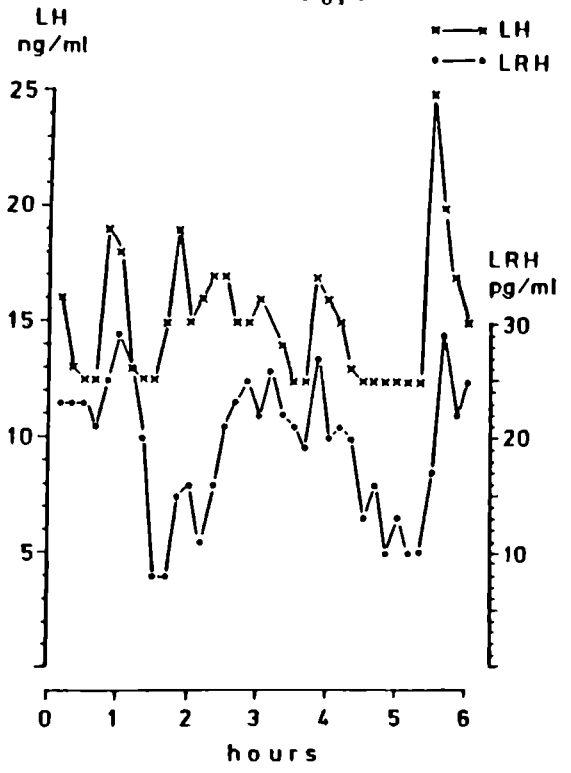


Figure 6.4 Continuous recording of plasma immunoreactive LH and LRH levels in male volunteer (IIGD).

Results from an experiment with a male volunteer are shown in figure 6.4. Immunoassayable LRH levels vary in a pulsatile manner between 10 pg/ml and 30 pg/ml. We see several typical LH-episodes (spiking) with LRH levels showing a parallel assayable plasma LRH pattern in three LH-episodes during the six hour registration time. Figure 6.5 shows the results for six female patients, who were being seen in the endocrinological out-patient section in the course of fertility studies. All subjects show more or less typical LH-spikes, and in some cases the immunoreactive LRH peaks appear to correlate with a LH-episode, but on the whole there is no over-all correlation between the two hormones.

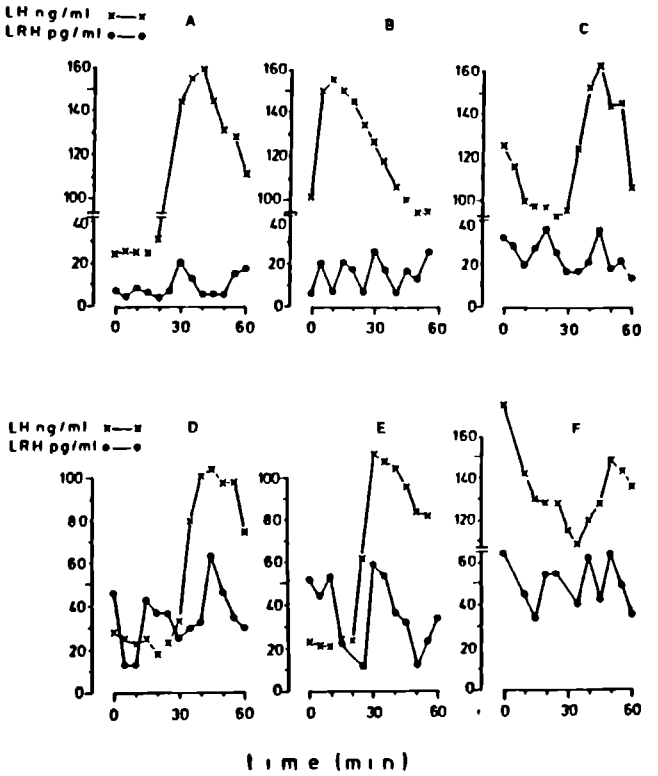


Figure 6.5 Continuous recording of plasma immunoreactive LH and LRH levels in 6 female patients.

## 6.7 Discussion

In studies of the stability of LRH in human plasma Saito et al. (1975) obtained almost identical results as in our study: a decrease during the first two hours at 20°C and a recovery of 14 % after 24 hours at this temperature. At 0°C the immunological activity was preserved for a few hours and was then gradually lost. In contrast to these findings, Jeffcoate et al. (1974) reported that enzymatic degradation in ovine plasma appeared to be a problem associated with the radioimmunoassay of thyrotrophin releasing hormone (TRH) but not with that of LRH. Nett & Adams (1977) studied the immunoreactivity of LRH in serum from a number of species. Pooled sera showed a

disappearance of immunologically active exogenous LRH in a time and temperature-dependent manner. The rate of disappearance varied from species to species with the half life ranging from 15 minutes in equine serum to 240 minutes in ovine serum The kinetics of the disappearance process suggests that it is enzymatic in nature This is further supported by the fact that addition to the sample of enzyme inhibitors such as Trasylol and Benzamidine results in higher levels of LRH (Aksel & Glass, 1979)

The non-specific inhibition of the binding of radiolabeled LRH to antibody has also been noted by other investigators Arimura et al (1973) could show that the effect is dose related They also reported that upon re-assaying a sample with apparently high LRH levels, while compensating for the serum effect by adding serum to all tubes, the sample then showed little or no LRH present This type of effect could explain the extremely high plasma levels (up to several ng/ml) reported by some investigators (e g Kerdelhue et al 1973) who neither compensated for the plasma effects nor extracted the plasma samples

Arimura et al (1974) showed that the non-specific inhibitory activity of serum or plasma on the binding in the LRH-radio immunoassay cannot be extracted in ethanol or aceton-water mixtures but that exogenous LRH can be recovered quantitatively in the extract They also confirm our findings that the non specific interfering material fluctuates from individual to individual and from time to time in the same individual (Arimura & Schally 1976) Further evidence for the necessity of employing an alcohol extraction prior to radioimmunological measurement of plasma LRH was presented by Jonas et al (1975) These authors showed that overnight storage at 37°C of ovine plasma containing high levels of immunoreactive LRH material and/or added synthetic LRH destroyed all exogenous LRH, while the endogenous activity remained largely intact. This means that the endogenous LRH like activity could not be identical to the (exogenous) decapeptide as the latter is broken down by enzymes present in plasma and the former not. The endogenous LRH could be present in a form protected for enzymatic action but this seems rather unlikely. More evidence for the dissimilarity of the two immuno-activities was given by the fact that methanol extraction of the samples removed the exogenous LRH but not the endogenous immunoreactive material

Concentrations of LRH in the peripheral circulation are likely to be very low and the background values in the assay due to non-specific plasma effects may very well hide any LRH that is present Elimination of such effects must therefore be considered very important. A very high specificity is also extremely important in order to obtain meaningful results. Considering our results, presented

in this and the preceding chapter 5, both these requirements seem to have been met in our LRH radioimmunoassay.

Failure to find elevated plasma LRH after injecting oestrogen-primed ovariectomized rats with progesterone may be due to pulsatile secretion of endogenous LRH similar to the secretion of LH, resulting in maintenence of basal LH levels. A sample may thus reflect only one moment in a fluctuating pattern, so that physiologically meaningful measurements cannot be done with this way of sampling. In this context it should be noted that the half-life of LRH in general circulation is much shorter then the time between peak levels of LH and FSH after stimulation by LRH (see chapter 8). This indicates that the presence of unchanged LRH in the circulation may not necessarily coincide with the time of maximal gonadotrophin release.

A number of investigators have reported low or non-detectable levels in peripheral plasma. Rosenblum & Schlaff (1975) found levels below 5 pg/ml for most normal individuals. Exceptions were noted during midcycle and in normo-gonadotrophic patients with amenorrhea, where levels up to 40 pg/ml were noted. However, they, and many others, used random sampling for these studies, and thus it is quite possible that the difference between the patient groups is a result of infrequent sampling. This kind of variation of immunoreactive LRH levels was also seen in our studies over 6 hours in one subject, confirming the need for a different, preferably continuous, sampling method when studying hormones such as LRH.

Our results obtained with continuous sampling indicate that LRH is in fact secreted in an episodic manner. Unfortunately, our studies offer no proof that the observed patterns are the result of episodic hypothalamic activities. However, over the years evidence for the hypothalamic origin of the pulsatile LH release, and thus for a pulsatile LRH secretory pattern has accumulated and its existence can hardly be disputed. Centrally acting drugs such as phenobarbitone (Blake, 1974) and nicotine (Blake, Norman & Saywer, 1974) can inhibit LH-spiking in ovariectomized rats and this inhibition can be overcome by administration of LRH. More direct evidence was obtained with a LRH radioimmunoassay by Nett, Akbar & Niswender (1974) who measured between 15 and 90 pg/ml immunoreactive LRH in ewes. As in our studies rhythmic oscillations in LRH were noted, but there was no apparent correlation with the LH release patterns.

Using the same radioimmunoassay (Nett, Akbar & Niswender, 1974; AS3 in chapters 4 and 5) and a special technique for collecting portal blood, Carmel, Araki & Ferin (1976) detected significant fluctuations of LRH in Rhesus monkeys. Peak concentrations of up to 800 pg/ml were observed with a peak to peak time of 1 - 3 hours. Bursts of immunoreactive LRH in concentrations up to 2000 pg/ml was

recorded in human portal plasma (Antunes et al. 1978). Simultaneous detection of (portal) LRH and (peripheral) LH surges has been achieved (Levine et al. 1982; Clarke & Cummings, 1982) as well as simultaneous detection of hypothalamic electrical pulses and LH pulses.

Thus, it seems that there is no doubt that hypothalamic LRH is released in a pulsatile manner. Whether our studies in fact picked up LRH secreted from the hypothalamus is another matter. Although the levels we and others measured are at a level where we could reasonably expect to find them, (based on back-calculations from the dose exogenous LRH needed to induce LH release: from a few pg/ml up to a few tens of pg/ml, chapter 9), more recent measurements in portal plasma give rise to estimates sometimes considerably lower. If we take the above cited 2000 pg/ml peak levels (Antunes et al. 1978), and consider that portal plasma will be diluted approximately 500 times upon reaching general circulation, the corresponding peripheral levels would be around 5 pg/ml. This upper level estimate would then correspond to our measured lower level. Other investigators found even lower levels immunoreactive LRH in the portal system: 40 - 160 pg/ml (Sarkar & Fink, 1979); 50 - 150 pg/ml (Ferin, Van Vugt & Wardlaw, 1984); 5 - 30 pg/ml (Clarke & Cummings, 1982).

One reason for the differing values might be found in that several different species were used for the measurements. It might be significant, in relation to our studies, that the highest values were found in humans. If we accept these values, the discrepancy between peripheral and portal LRH concentrations are reduced to a reasonable but still significant figure. Another factor that should be taken into consideration is the extra-hypothalamic occurrences of LRH. Although it was originally thought to be a specific hypothalamic releasing hormone, several other sources for the hormone have been found (Siler-Khodr & Khodr, 1978; Gibbons, Mitnik & Chieffo, 1975, Baram et al. 1977; Paull et al. 1981 and others). The extrahypothalamic functions of LRH are largely unknown although speculations are available in quantity. Be that as it may, it is conceivable that the peripheral immunoreactive LRH detected, in our studies and those of others, may not reflect hypothalamic activity but rather some other as yet undefined function(s). Yet, it is of course also possible that the peripheral immunoreactive LRH activity is of a mixed hypothalamic and extrahypothalamic origin. The question remains unsolved and awaits its solution by other means.

If we want to really prove that what we measure in plasma is the releasing hormone and nothing else, we would have to include other assays, relying on other principles such as bioassays or chromatographic motility. This has been done for the thyroid stimulating hormone releasing hormone, TRH. The same controversy as regards

peripheral hormone levels is also found there. Using immunological, chromatographic and enzymatic evidence it was possible to detect approximately 80 pg/ml in human peripheral blood (Mallik et al. 1980). Although the results seem rather conclusive, even in this case there is no proof for a hypothalamic origin of the releasing hormone activity.

## 6.8 Summary

In this chapter we have shown that direct measurement of LRH by means of radioimmunoassay has many pitfalls. Proteolytic enzymes can cause a loss of LRH. Other macromolecular components of can interfere and invalidate results. Non-specific interference can largely be eliminated by an alcohol extraction procedure that was developed and validated in this chapter. Continuous sampling is an essential tool when measuring LRH in circulation, as it seems to be secreted in a pulsatile manner and has a relatively short half-life. In the next chapter we will describe the measurement of LRH in tissues and in vitro.

# CHAPTER 7

## Measurements of the hormone in tissues and incubation media

### 7.1 Introduction

There are three reasons why we decided to extend the radioimmunological measurement of LRH to tissues. First, measurement of tissue concentrations of the hypothalamic releasing hormones is likely to be essential for the elucidation of their physiological role. As reported in chapter 6, only small amounts of releasing hormone are secreted into the hypothalamo-hypophyseal portal system, and these are diluted to a great extent upon entering the general circulation. This means that we can expect determination of releasing hormone content in the hypothalamus to yield more precise information about changes in production and release of these hormones.

Secondly, validation of a radioimmunoassay involves a comparison of a standard and its physiological counterpart. Parallelism of dose-response curves between unknown and standard is a requirement for a valid radioimmunoassay, but is in itself no proof of identity (Ekins, 1974). In the case of radioimmunoassays for hypothalamic releasing hormones the assay sensitivity is being pushed to the limit for measurements in the general circulation, so that parallelism cannot be investigated, much less be established. The only biological material with which we can hope to do this is a hypothalamic extract.

Thirdly, the mechanisms regulating hypothalamic LRH synthesis and release are not yet fully understood. It was generally accepted at the time when these studies were carried out that biogenic amines are involved in the processes. Studies of these systems commonly employ in vivo and in vitro methods. The latter often involve the incubation of hypothalamic fragments and the detection of releasing hormone activity by means of bioassays or radioimmunoassays. In the course of this study we decided to investigate whether it is possible to obtain some information about the involvement of biogenic amines in LRH release by means of a simple incubation technique and a more elaborate perifusion method.

7 2 Determination of LRH in tissues

7 2 1 LRH content of selected parts of the brain

Tissue extracts were obtained by means of extraction in hydro chloric acid For this purpose 20 male Sprague Dawley rats weighing 160 - 200 g were decapitated The hypothalamus (stalk median minence), posterior pituitary, pineal body and a part of the cerebral cortex were immediately excised and homogenized in 0 1 M HCl After centrifugation at 30,000 x g for 30 minutes in a refrigerated centri fuge, the extracts were neutralized with 1 N NaOH and recentrifuged The supernatants were subjected to LRH radioimmunoassay in tri plicate on five or more dose levels In addition, we assayed porcine posterior pituitary extracts, prepared in the same manner during earlier experiments and kept frozen ever since The LRH contents of the tissue extracts were calculated from the average of these data and expressed as ng LRH / tissue fragment

The median eminence extract was found to contain 7 10 ng (SE 0 9 ng) Figure 7 1 shows the inhibition curves obtained with the various extracts in the radioimmunoassay The inhibition curves obtained with synthetic LRH and median eminence extract are paral lel indicating that the median eminence contains large amounts of a material that is immunologically identical to the decapeptide Cerebral cortex and the porcine posterior pituitary extracts contained small but measurable amounts of radioimmunoassayable LRH activity, 0 07 and 0 45 ng respectively, whereas the pineal extracts and the rat posterior pituitary extract contained less then 0 005 ng per gland

7 2 2 Effects of gonadal steroid feed back on hypothalamic LRH content in male rats

Steroid feed back mechanisms are generally complex In gonadal feed back mechanisms there are inhibitory as well as stimulatory effects on the pituitary, and feed back signals may also involve the hypothalamus In addition to gonadal feed back signals we must also consider the existence of short feed-back route from the pituitary In order to cast some light on this, we investigated in a pilot experi-ment, the effect of castration and hypophysectomy on hypothalamic LRH content Castration reduced the LRH level to about half of that in the control untreated group The same was true for the hypophys-ectomized rats These rats, deprived of essential growth factors such as growth hormone, failed to develop normally and weighed at the end of the experiment only half as much as the other animals Therefore, we decided to investigate whether hypothalamic LRH

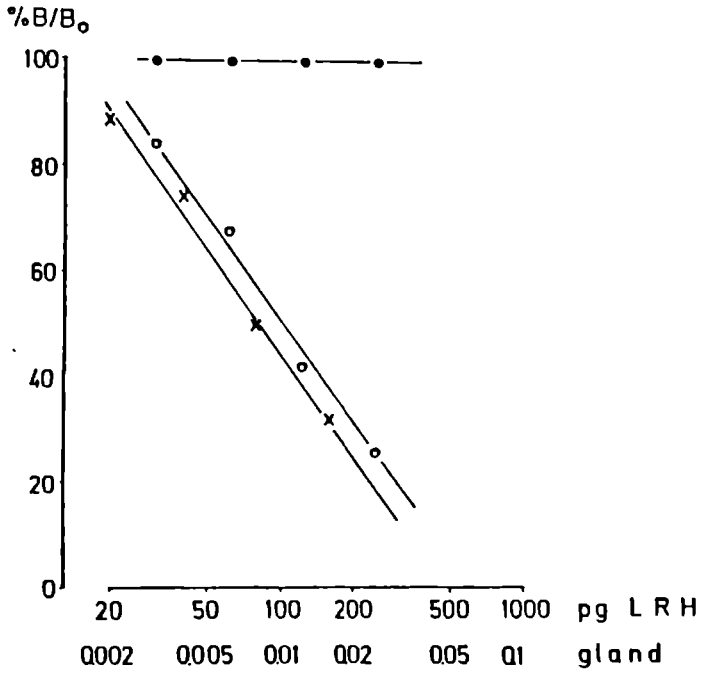


Figure 7.1: Inhibition curves obtained in the LRH-radioimmunoassay by synthetic LRH (x--x), median eminence extract (o--o) and extracts of posterior pituitary, cerebral cortex and pineal body (•--•). Tissue extracts are expressed as decimal fraction of gland or hypothalamus.

content is related to body weight. Male Wistar rats (FW95) were obtained at different ages with body weights ranging from 120 to 400 g. Groups of 10 rats were decapitated and the median eminences removed, extracted and assayed for LRH content as described above in section 7.2.1 The results shown in figure 7.2 indicate a positive correlation between body weight and hypothalamic LRH content.

In order to investigate the effect of gonadal steroid feed-back we divided 50 male Wistar rats were divided into five groups of 10 rats each: 1. an untreated control group, 2. animals subjected to transaurical hypophysectomy by means of suction through a capillary, 3. animals that were orchidectomized, 4. animals given a subcutaneous injection of 50 mg testosterone, 5. orchidectomized animals receiving 50 mg testosterone. All animals were decapitated 5 weeks after these

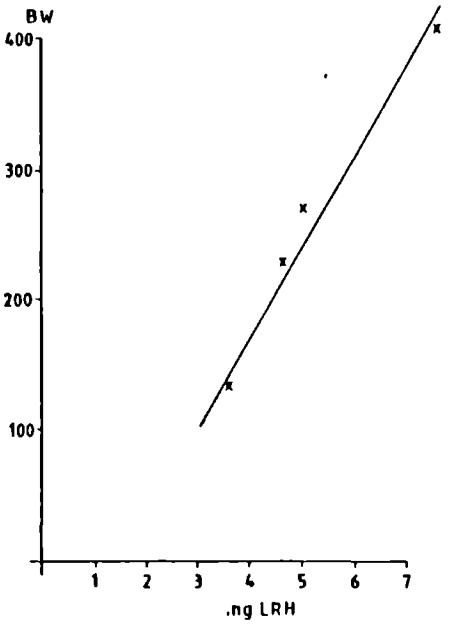


Figure 7.2: Correlation between body weight and hypothalamic LRH content in male rats of various ages.

treatments and the hypothalamic LRH content was determined as before. The results are shown in table VII.1

Table VII.1 Hypothalamic LRH content in male rats with different states of gonadal steroid feed-back.

| treatment group | ng LRH per med. emin. | ng LRH per 100 g BW |
|---|---|---|
| control | 7.7 +/- 1.4 | 2.3 +/- 0.4 |
| hypophysectomy | 3.6 +/- 0.8 | 2.3 +/- 0.5 |
| orchidectomy | 3.7 +/- 1.0 | 1.0 +/- 0.3 |
| testosterone | 8.4 +/- 1.1 | 2.8 +/- 0.4 |
| orchidectomy + testosterone | 6.9 +/- 1.1 | 2.2 +/- 0.4 |

averages with SD

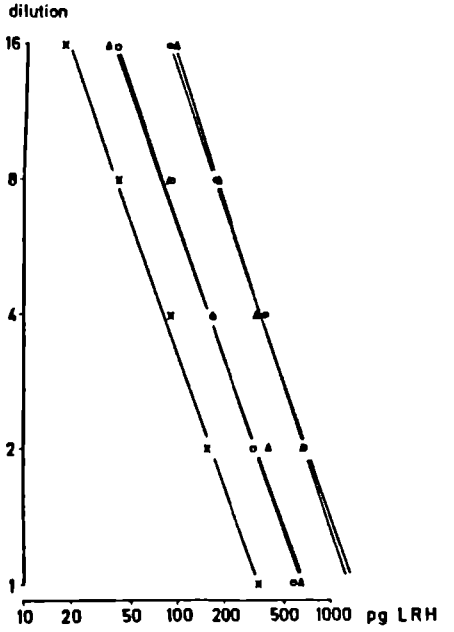


Figure 7.3: Inhibition curves obtained in the LRH-radioimmunoassay with synthetic LRH (x--x) and median eminence extracts from rats subjected to orchidectomy (o--o), hypophysectomy (open triangles), testosterone replacement (filled triangles) and control animals (•--•).

It is obvious that the testosterone treatment alone had no effect on hypothalamic LRH content, whether calculated per rat or per 100 g body weight. In the case of castration and hypophysectomy there is a significant reduction to about half in both these cases when considered per rat. However, the effect in the hypophysectomized group disappears when considered per 100 g body weight. The testosterone substituted orchidectomized rats had a hypothalamic LRH content comparable to the control group, indicating that the reduction was due to the absence of testosterone feed-back in these rats.

The data from the LRH radioimmunoassay in the above experiments are also plotted in figure 7.3. The paralell lines obtained by plotting radioimmunologically measured LRH against the dilution factor shows that there is a high degree of immunological identity between the different hypothalamic extracts.

### 7.3 Determination of LRH in tissue incubation media

#### 7.3.1 Incubation of rat hypothalami

We investigated the possibility of modifying the LRH release from hypothalami in a simple incubation system, by addition of steroids and biogenic amines in order to obtain information about the involvement of these compounds in the release process. Such a technique would then enable further investigations of the regulatory mechanisms of LRH release Female Wistar rats (300 g body weight) were decapitated The hypothalami were immediately excised and five each placed in 2 ml Krebs Ringer bicarbonate glucose solution (KRBG) This solution contained 3% bovine serum albumin, was adjusted to pH 7.4 and then gassed carbogen (95% carbondioxide/ 5% oxygen). In these studies the hypothalamus was defined as the tissue lying between the optic chiasm on the anterior side, the hypothalamic sulci on the lateral sides, the mammilary bodies on the posterior side, and the third ventricle on the superior side There were four treatment groups 1. controls, 2 addition of 5 µg dopamine, 3. addition of 100 µg oestradiol, 4 addition of both dopamine and oestradiol The oestradiol was dissolved in 5 µl ethanol, which amount of solvent was also added to the control incubations For each treatment five incubations were carried out After 15 mins preincubation the medium was discarded and replaced with 2 ml fresh medium The hypothalami were then incubated for 2 hours at 37°C under gassing with carbogen Incubation was stopped by deproteinization through the addition of 8 volumes of ethanol After centrifugation the supernatants were extracted as described in section 6 3. The results of the subsequent LRH radioimmunoassay are presented in Table VII.2.

Table VII.2 LRH released in incubation media in the presence and absence of dopamine and oestradiol

| Treatment | LRH pg/ml medium |
|---|---|
| control | 176 +/- 19 |
| dopamine | 204 +/- 24 |
| oestradiol | 185 +/- 14 |
| dopamine + oestradiol | 227 +/- 14 |

Averages with SE from 5 incubations with 5 hypothalami each

The average LRH levels are higher in the presence of both dopamine and oestradiol, but the difference is not statistically significant ($0.05 < P < 0.1$).

Another experiment along these lines was carried out to test the effects of norepinephrine and dopamine on LRH release. Hypothalami were obtained from male rats weighing about 150 grams. The experimental details were identical to the above experiments with the only exception that the final incubation time was only 5 minutes. The short incubation time was chosen on the assumption that proteolytic enzymes might be present and degrade part of the released LRH. A wide range of concentrations of the two biogenic amines was used in the absence of any reliable information of the possible effects. Considering the wide range of amine concentrations that was employed, we did not include a "zero concentration". The results are shown in Table VII.3

Table VII.3 LRH content of incubation media in the presence of dopamine and norepinephrine.

| catecholamine | concentration (µg/ml) | LRH (pg/ml) |
|---|---|---|
| norepinephrine | 0.001 | 57 +/- 29 |
| -"- | 0.01 | 108 +/- 46 |
| -"- | 0.1 | 59 +/- 32 |
| -"- | 1.0 | 40 +/- 16 |
| -"- | 10.0 | 53 +/- 15 |
| dopamine | 0.001 | 24 +/- 7 |
| -"- | 0.01 | 78 +/- 33 |
| -"- | 0.1 | 39 +/- 15 |
| -"- | 1.0 | n.d. |
| -"- | 10.0 | n.d. |

Averages with SE from 5 incubations per dose-level with 5 hypothalami each, n.d. = not detectable.

Both catecholamines seem to increase the LRH release at an intermediate concentration of 0.01 µg/ml. Unfortunately, the variations within the groups were again too large to obtain statistically significant effects.

Several attempts were made to improve the reproducibility and stabilize the system. This included lowering the incubation temperature from 37°C to room temperature (20°C), and varying the preincubation and incubation times. None of these changes gave better reproducibility than the above experiments. We concluded that static incubation of hypothalami as described above does not yield sufficiently reproducible data to permit studying the regulation of LRH release from the hypothalamus.

7.3.2 Perifusion of rat hypothalami

As static incubation of hypothalami did not seem to be a suitable technique for studying the regulation of LRH release, we decided to set up a perifusion system. This would continuously provide the hypothalami with fresh incubation medium, thus removing waste products, offering fresh nutrients and allowing frequent sampling. Carbogen gassed KRGB - 3 % BSA is passed through a heat exchanger and oxygenator by means of a peristaltic pump. The whole system is kept at 37°C in a thermostated water bath. After removing gas bubbles in a debubbling device, the medium is passed through a 10 µm membrane into a perifusion chamber. After leaving the chamber through another membrane the medium is collected on a fraction collector. The collection tubes are kept on ice and contain the ethanol required for the extraction procedure as described in section 6.3. As soon as a tube has recieved the desired amount of medium, it is mixed on a Whirlmix for 15 seconds and centrifuged. This is followed by determination of radioimmunoassayable LRH as described earlier. Flow rates are kept between 0.10 and 0.25 ml/minute and the collection time for each sample is between 3 and 12 minutes. Before each experiment the whole apparatus is rinsed with the perifusion medium to coat it with albumin and bring it to the desired temperature. Standard curves for the subsequent radioimmunoassay are prepared in the same medium and the standard samples are extracted simultaneously with the experimental samples as described in section 6.3. In initial experiments we investigated whether LRH can be recovered from the perifusion medium. LRH was injected as a pulse into the tubing entering the perifusion chamber, which contained one hypothalamus. About 2/3 of the injected quantity (120 pg) could be recovered from the perifusate. In a second experiment medium containing 10 pg LRH / ml was prepared and samples were collected in three different ways: the first sample was kept on ice without extraction, the second sample was extracted with ethanol (section 6.3) and the third sample was extracted in the same manner with methanol. After passage of the medium through the perifusion chamber with a hypothalamus we could detect 8.6 +/- 0.8 pg / ml without extraction, 13.8 +/- 1.7 pg / ml in the ethanol extracted samples and 14.0 +/- 1.2 pg / ml in the methanol extracted samples (5 samples each, assayed in triplicate, mean +/- SD). Without extraction we find - as expected - about 60 % of the LRH obtained in the extracted samples. Furthermore, the results from the extracted medium samples indicate that a small amount of LRH (ca. 6 pg/ml) is added to the medium during its passage through the perifusion chamber, presumably due to release by the hypothalamus.

Basal LRH release into the perifusate was further investigated by a perifusion of two hypothalami for 6 hours. The hypothalami in this and the following experiments were obtained from male rats

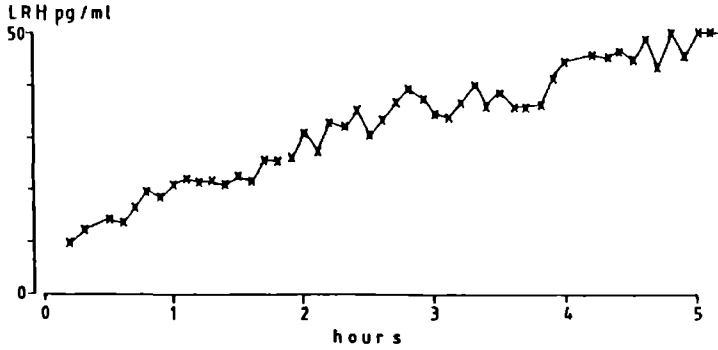


Figure 7.4: Basal release of immunoassayable LRH from two perifused rat hypothalami.

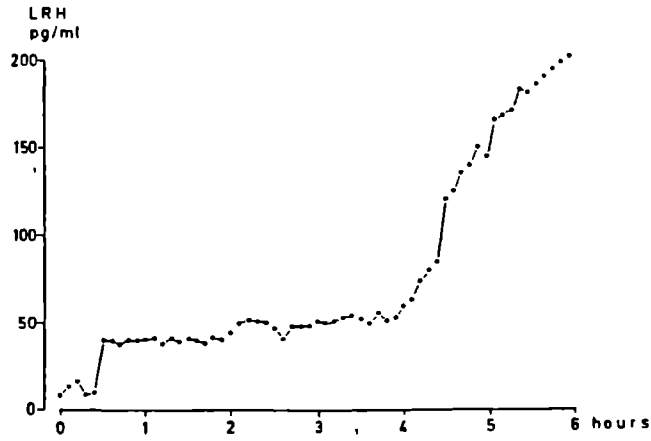


Figure 7.5: Basal release of immunoassayable LRH from two perifused rat hypothalami.

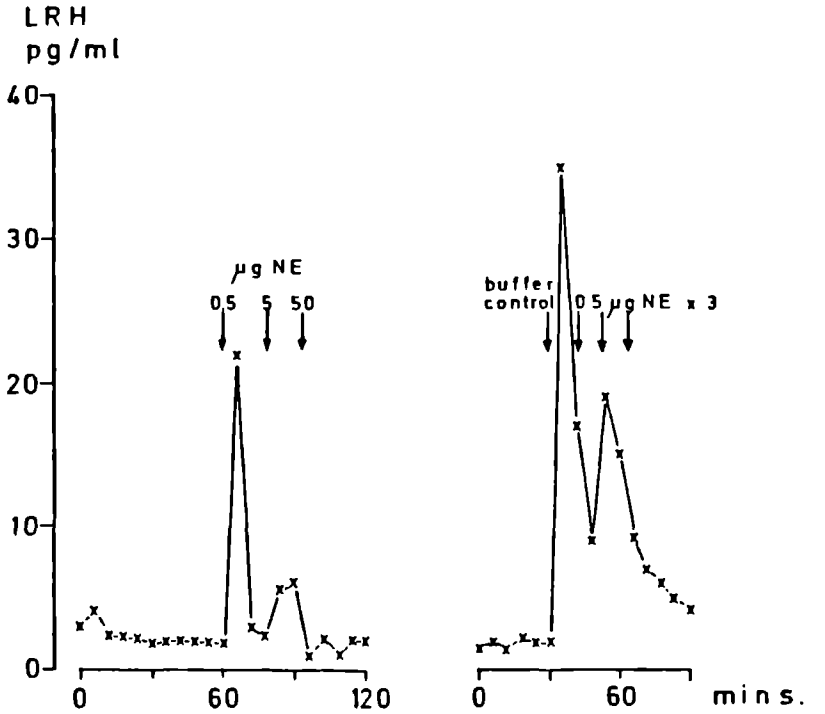


Figure 7.6: Immunoreactive LRH in perifusate from 2 hypothalami subjected to norepinephrine pulses.

weighing between 150 and 250 g. The rats were decapitated after stunning without anesthesia. incubation temperature was 37°C and the collection time for each medium fraction was 6 minutes. Figure 7.4 shows that LRH release can be detected in this way. There is a steady increase from 10 - 15 pg/ml to a level of 50 - 60 pg/ml at the end of the 6-hour perifusion period. A slightly different picture is seen in figure 7.5. In this experiment there is a steady release of ca 50 pg/ml 4 hours, after which tissue decay sets in and larger amounts of LRH are liberated into the medium.

Numerous attempts were made to stimulate the release of LRH into the medium by means of catechol administration. A few typical examples are shown above. In the experiment shown in figure 7.6 (left), pulses of norepinephrine were injected into the perifusion medium just prior to where the tubing is entering the perifusion

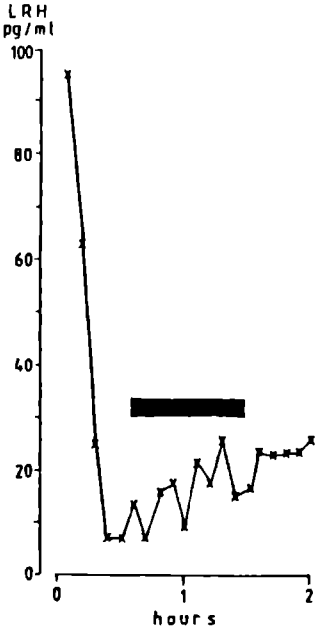


Figure 7.7: Immunoassayable LRH in perfusate from 2 hypothalami subjected to continuous exposure to norepinephrine.

chamber. The first pulse consisted of 0.5 µg norepinephrine in 100 µl KRGB, injected slowly over a 3 minute time period. Ten minutes later this was followed by a 5 µg injection, and thereafter by a 50 µg injection. A sharp increase in perifusate LRH levels can be seen after the first pulse of norepinephrine, while smaller increments in immunoassayable LRH were detected after the second and third pulses. The experiment was repeated, but this time with a pulse of KRBG followed by three 0.5 µg norepinephrine pulses. The results, shown in figure 7.6 (right), suggest that the LRH increases detected in this and the previous experiment are more likely due to an injection artifact, rather than by a drug-induced release.

In view of these results we decided to avoid such disturbances in the medium flow and to administer the norepinephrine continuously. This also permitted maximal stimulation of the system. This was achieved by switching to a medium containing 10 µg/ml catecholamine after a 15-minute pre-perifusion period with normal KRGB. Results are

shown in figure 7.7. In this experiment the initially high LRH levels are followed by a drop to 10 - 25 pg / ml. No significant reaction to norepinephrine treatment during 1 hour is observed. The experiment was repeated three times with virtually identical results were obtained. Norepinephrine doses of 5 and 100 µg/ml were also employed in this manner, but again the results did not show any response to the treatment.

## 7.4 Discussion

### 7.4.1 Tissue LRH content

As expected,the hypothalamus was found to be an excellent source of LRH. The extraction method yielded LRH in amounts that are comparable to those found by other investigators. The importance of using a proper extraction method is emphasized by the results of Shin & Howitt (1974). They found that using a buffer extraction and omitting a boiling step reduced the recovery to 10 %. This suggests that the LRH is stored in a inaccessible form to simple buffer extraction. The use of acid, organic solvents or boiling is essential.

Ramirez et al. (1975) found 5.5 +/- 0.9 ng per rat hypothalamus with a bioassay (Ramirez & McCann 1963) and 5.1 +/- 0.3 ng (mean +/- SE) with a radioimmunoassay. These values (for 200 g male rats) are very similar to our data (4.5 ng). As in our studies, these investigators could show a parallelism between standard and tissue sample in the radioimmunoassay system. This could also be established for human hypothalamic extracts by Mortimer et al. (1976). Jeffcoate et al. (1974b) reported hypothalamic LRH contents between 0.7 and 5.8 ng LRH in the rat, 8 - 12 ng in the rabbit and 0.030 - 1.0 ng in the chicken. All extracts exhibited dilution curves that are parallel to those for synthetic LRH. These values appear to confirm the relationship between animal size and hypothalamic LRH content, which we have demonstrated for hypothalami from rats of different weights. Araki et al. (1975) presented almost identical results. However, they used only rats up to the lowest weight investigated by us. The dependence of hypothalamic LRH content on body weight needs to be taken into account when comparing results from experiment with rats of different sizes. It is obvious that the results of the hypophysectomy treatment can only be interpreted correctly when body weight is taken into consideration.

There is no doubt that the regulation of gonadotrophin secretory patterns is under some type of steroid feed-back control. After gonadectomy, both sexes in many species will show elevated gonadotrophin levels (Gay & Midgley, 1969; Yamamoto, Diebel & Bogdanov,

1970; Blackwell & Amoss, 1971). Treatment of ovariectomized monkeys with physiological levels of oestradiol rapidly depresses plasma LH levels and interrupts the pulsatile release pattern (Yamaji et al. 1972). Similar results have been obtained in man (Yen et al. 1972). In early experiments Piacsek & Meites (1966) found an increased bioassayable LRH content of the rat hypothalamus after castration, which could be restored to normal by administration of testosterone propionate. Consequently, we were slightly surprised when the effect of castration in our studies turned out to be a decrease in hypothalamic LRH content by approximately 50 %, which change could be reversed by testosterone treatment. Similar reductions were also observed by Shin & Howitt (1975), who showed that hypothalamic LRH initially increases for the first 48 hours after castration, followed by a gradual decrease to approx. 50 % of the normal levels after about one week. This was also observed by Baker & Dermody (1976), Rotztein et al. (1977) and Martini (1983). All these investigators used rats and radioimmunoassays. However, similar results were obtained in mice by Hegeman et al. (1983). In women reduced hypothalamic LRH levels are found after ovariectomy and in the menopause Parker, Parker & Porter, (1984). This indicates that such mechanisms are operative in various species.

Mouguilevsky et al. (1975a), measuring the incorporation rate of tritiated tyrosine into peptides with LRH-activity, found a higher rate in castrated rats than in untreated rats. This indicates that removal of the gonadal steroid feed-back leads to an increased synthesis of LRH, which is in apparent contrast to our studies and those of others. However, we must recognize that when hypothalamic LRH content is measured, the results do not necessarily reflect the rate of synthesis of the releasing hormone. It is more likely that in fact the difference between synthesis and release into the portal system is measured. Thus, it is conceivable that the decrease observed by us is the result of an increased release that is not paralleled by the same increase in the synthesis of the peptide. This suggestion is supported by the results of Mouguilevsky et al. (1975b), who by means of the Ramirez-McCann bioassay showed that hypothalami from castrated rats have a higher rate of synthesis and a lower LRH content.

White et al. (1974) reported very large amounts of LRH in ovine, bovine and porcine pineal glands. These studies were carried out with a radioimmunoassay as well as a bioassay. The pineal gland was found to contain 4 - 10 times more releasing hormone than the hypothalamus from the corresponding species. These unexpected findings prompted us to measure the LRH content of the rat pineal gland, which we found to contain less than 0.005 ng LRH. It seems unlikely that this would merely be due to species differences. Indeed, other investigators using the same methods as we did (Gradwell, Millar &

Symington, 1976; Duraiswami et al. 1976) could not confirm the high values of White and collaborators for bovine pineal glands.

### 7.4.2 Incubation studies

The results of the incubation experiments suggest that there are factors interfering with the assay. One obvious source of trouble is proteolytic degradation of LRH by hypothalamic peptidases. The existence of such enzymes have been demonstrated in the soluble fraction of hypothalamic tissues (Griffiths & Hooper, 1973; Griffiths et al. 1975, Koch et al. 1974). The levels seem to be inversely correlated with the LH levels and possibly with the LRH activity. The peptidase activity was shown to be able to degrade LRH, resulting in a loss of both immunological and biological activity. This was the rationale for trying shorter incubation times. The use of enzyme inhibitors was rejected, since these compounds are often very unspecific.

The later findings of Negro-Vilar, Ojeda & McCann (1979) offer another explanation for the absence of a response in our incubation studies. Their results indicate that the choice of the hypothalamic region is quite critical when incubating hypothalamic tissue. They only found dose-dependent stimulation by norepinephrine and dopamine when incubating the median eminence alone, but not when surrounding tissue was present. They suggest that LRH release from the median eminence is inhibited by neurons with cell bodies in the surrounding structures. This has been confirmed by other recent studies where the median eminence was also responding much better than the medial basal hypothalamus (Gambacciani, Yen & Rasmussen, 1986). This speaks in favour of a neural system, present in the intact hypothalamus only, which is capable of inhibiting LRH release. It thus appears that in our desire to include the complete hypothalamic structure for LRH release studies so as to have a system resembling the in vivo situation as closely as possible, we may have in fact included inhibiting functions.

Other investigators were equally unsuccessful in provoking a change in LRH release in vitro by means of biogenic amine stimulation. Kao et al. (1977) testing the effects of acetylcholine, norepinephrine, dopamine and serotonin in a perifusion system, were also unable to find any effects. The levels of immunoassayable LRH in the medium were close to those in our experiments. Yet other investigators could show dose-dependent stimulation of LRH release from hypothalami in vitro with very low doses of catecholamines (less than 1µM), whereas higher doses did not lead to LRH release (Ojeda, Negro-Vilar & McCann, 1979). The doses used in our perifusion experiments were according to their results, too high to be effective: a

0 5 microgram pulse the lowest concentration used by us, would lead to nearly millimolar levels The same is true for the experiments of Kao et al (1977) In our static incubation experiments, we observed elevated LRH levels, albeit not statistically significant, at the lower catecholamine concentrations In other words, it is quite possible that the doses employed in many of our experiments did not permit physiological LRH release It is probably fair to state in the seventies when we carried out our experiments, there was a tendency to aim for maximal stimulation Since then the scientific community has come to realize that the use of "pharmacological' concentrations and even the higher ranges of physiological concentrations can lead to misleading results

Although there is solid evidence for a stimulatory role of norepinephrine in the release of LRH from the hypothalamus (Rance et al 1981, Ojeda et al. 1982, McCann et al 1979, McCann, 1983a and b), evidence for suppression have also accumulated (Leung et al. 1982, Parvizi & Ellendorf, 1982, Donoso et al 1971, Gallo & Drouva, 1979, Gallo, 1981) The same is true for the other putative synaptic transmitter, dopamine, where stimulatory effects (Schneider & McCann, 1969, 1970a, 19870b and 1970c) as well as suppressive effects (Fuxe & Hoekfelt, 1969, Gnodde & Schuiling, 1976) have been seen Later studies have shown that both effects are equally possible and that this probably depends on the steroid environment (McCann, 1983a & 1983b, Vijayan & McCann,1978a and 1978b) In our studies the steroid enviroment (with one exception) was not controlled beyond the selection of animals, and it is thus conceivable that this factor contributed to the results In the one instance where oestradiol levels were altered, we unfortunatly used again a dose that was far beyond physiological.

The importance of selecting the correct steroid environment for LRH release studies is demonstrated by the following experiments. The response to dopamine stimulation of hypothalamic tissue in vitro is one order of magnitude lower for an ovariectomized rat, which is has not been primed with an oestrogen injection (Negro-Vilar et al. 1979) There is a higher "basal" LRH release by superfused rat hypothalami from oestrogen-primed ovariectomized rats that were treated with progesterone prior to the experiment (Ramirez et al 1980) Furthermore, progesterone can induce LRH release from such preparations in vitro, but only if the rats are primed with oestrogen (Miyake et al. 1982, Kim & Ramirez, 1985)

Another factor that might determine whether the action of the neurotransmitters will be stimulatory or not is the mode of adminis tration Continuous infusion of norepinephrine into the third ventricle of rats can suppress LH output after an initial peak (Gallo, 1982) In contrast, when the amine was given at spaced intervals, large LH-

responses were noted for each norepinephrine pulse. Similar results are seen when stimulating the LRH output in vitro by means of progesterone administration to hypothalami derived from ovariectomized, oestrogen primed rats. Only a pulsatile but not a continuous mode of infusion was effective in stimulating LRH release. There was a latency period of approximately 1 hour (Kim & Ramirez, 1982).

The lack of effect with high doses and prolonged exposures can be explained by phenomenon of desensitization or refractoriness, i.e. a decreased response to repeated or continuous stimulation. This has been noted for a large number of cellular processes and it can either be acute or chronic. The acute desensitization is developing rapidly and is also rapidly reversible, the chronic one is slow both in onset and reversal. The acute effects possibly involves a decreased receptor function, whereas the chronic change often involves a loss of receptors or down regulation (Chang & Cuatrecasas, 1983). This altered response to stimulation appears to play an important role in the regulation of hormone action.

There are other ways of stimulating the release of LRH from hypothalamic tissue in vitro. Using a static incubation system, Brideli & Snyder (1978) measured approximately 10 pg LRH / ml medium, which value was increased by 200 - 500 % upon depolarizing the cell membranes by increasing the potassium concentrations to 60 mM or adding ouabain to the medium. Exclusion of calcium from the media prevented this increase. The isolated median eminence from ovariectomized rats, primed with oestrogen, responded to superfusion of 30 mM potassium enriched medium with reproducible, rapid and reliable LRH outputs (Kim & Ramirez, 1982). This potassium-evoked release depends on the temperature and calcium levels (Hartter & Ramirez, 1980). Furthermore, LRH release evoked in this manner can be altered by pretreatment with progesterone (Drouva et al. 1983).

Thus, it seems that LRH release from the hypothalamus depends on membrane depolarization and a resulting calcium influx into the cells. This mechanism is found for a growing number of hormones in many tissues, and has been substantiated by many investigators. Recently, Drouva et al. (1984) have shown the involvement of calmodulin and a calcium/calmodulin dependent protein kinase in the calcium dependent, potassium evoked LRH release from hypothalami in vitro.

In view of the experiments carried out in recent years, it is obvious that simple in vitro experiments in the manner described here can only yield meaningful information when a number of conditions are fulfilled. Most of them were not known at the time we did our in vitro experiments and thus the results are a consequence of, and demonstrating the need for employing the correct mode of agonist

introduction and experimental model. With the wisdom of hindsight we can visualize a number of approaches that could have yielded better information about the regulation of LRH release from the hypothalamus. However, we had to use the available information at that time and the results were as presented here.

## 7.5 Summary

In this chapter we have investigated the distribution of LRH in various parts of the rat brain. The highest concentrations were measured in the stalk median eminence. Low or non-detectable levels of immunoassayable LRH were found in the posterior pituitary, pineal gland and cerebral cortex. We could achieve one important aspect of radioimmunoassay validation by establishing parallel dose-response curves for standard and experimental samples. A relationship between body weight and hypothalamic LRH content was established. Removal of gonadal steroid feed-back led to a reduction in LRH content, probably as a result of a change in the ratio of biosynthesis and release of the releasing hormone. Two in vitro systems for possible use in investigations of regulation of LRH release were investigated: a simple static incubation system and a perifusion method. Basal release of LRH could be measured, but no significant responses to biogenic amines could be detected, probably because the entire hypothalamus and too high levels of biogenic amines were used.

# CHAPTER 8

## Studies with LRH in human subjects

### 8.1 Introduction

The availability of synthetic LRH (Matsuo et al. 1971, Geiger et al. 1971) provided the means to develop a rapid and efficient test of the capacity of the pituitary gland to respond to hypothalamic stimulation. In this manner, it became possible to detect degrees of functional capacity ranging from absolutely insufficient via adequate to overresponsive reactions. This technique could be very useful for evaluating the pituitary-hypothalamic axis in endocrine disorders of the gonadal-pituitary-hypothalamic system. Our research group was fortunate in receiving as one of the first in Europe samples of synthetic LRH. However, this meant that we had to carry out all experiments without any knowledge of how to use this new hormone in practice. Consequently, when we conducted the clinical studies with LRH, we had to determine first the time required for the pituitary to respond to LRH by measuring plasma LH and FSH levels in women and men. We also had to establish the minimum effective dose of LRH before we could carry out investigations of the pituitary capacity in a variety of endocrine disorders affecting the human reproductive system.

Early studies indicated that LRH can be used to induce release not only of LH but also of FSH in a number of species, including man (Schally et al. 1971; Schneider & Dahlen, 1972a, 1972b,; Yen et al. 1972). It is also possible to induce follicular growth and ovulation by employing LRH to elevate the circulating LH and FSH levels (Zarate et al. 1972; Schneider & Dahlen, 1973c; Keller, 1973; Bohnet et al. 1974). As these studies indicated that relatively long treatment schedules are necessary, it seemed desirable to find an easy and reliable way for self-administration. Intravenous, intramuscular and subcutaneous injection as well as oral and nasal administration lead to release of LH and FSH (Amoss et al. 1972, Mortimer et al. 1974, London et al 1973). Hoechst AG developed a spray for self-administering peptides or other similar materials into the nasal cavity. When reasonable amounts of synthetic LRH became available, we were given the oppurtunity to test the spray and evaluate the possibility of using it for chronic LRH therapy.

Considerable variation has been observed in sensitivity to a test dose of LRH. Steroid pretreatment can alter the pituitary sensitivity (Matsuo et al. 1971). During the human menstrual cycle and the rat oestrous cycle, responsiveness varies according to the steroid environment (Yen, 1977; Goodman, 1978; Fink,1979). A pulsatile fluctuation of

basal LH release of variable frequency and amplitude has been related to the different phases of the menstrual cycle (Schally et al. 1971; Yen et al. 1972; Santen & Bardin, 1973; Brody et al. 1982; Bachstrom et al. 1982; Liu & Yen, 1983; Veldhuis et al. 1984; Crowley et al. 1985). Both frequency and amplitude seem to be modulated by ovarian steroids. However, changes in sensitivity to LRH of the gonadotrophic cells could also be due to limitations in reserve capacity or a refractory period of the pituitary. In this context we investigated the behaviour of the human pituitary in response to repeated LRH stimulation. Our results led us to postulate the existence of a refractory period of the anterior pituitary to LRH stimulation.

## 8.2 Materials and methods

Eugonadal volunteers and patients with a variety of endocrine disorders were fitted with a jugular vein catheter. Blood was withdrawn by a peristaltic pump at 3-7 minute intervals in heparinized centrifuge tubes kept on ice. Samples were centrifuged immediately and the plasma snap-frozen and stored at -30°C. Plasma LH and FSH were measured by radioimmunoassay employing a double antibody technique (Dahlen, Keller & Schneider, 1974). LH was iodinated according to the method of Greenwood, Hunter & Glover (1963), utilizing highly purified material of human origin (LER-960 and a similar preparation from Dr Schams, University of Munich, Weihenstephan). LH antiserum nr. 391 was a gift from Dr. A.R. Midgley, University of Michigan, Ann Arbor, USA. The materials for the FSH radioimmunoassay were supplied by the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md, USA. In both assays 2nd International Reference Preparation of Human Menopausal Gonadotrophin (2nd IRP-HMG) was used as a standard. Anti-rabbit-gamma-globulin was obtained by immunization of sheep with Cohn fraction II of normal rabbit serum in Freund's complete adjuvant (see also section 4.3.2). The precipitated radioactivity was measured in a LKB-Wallac Gamma Scintillation Counter using a 3" x 3" Be-coated crystal. The inter-assay error (coefficient of variation) was 11.7% for the LH-assay and 9.4 % for the FSH-assay as determined by replicate determinations of a control plasma (average binding percentages 35 and 20%, respectively). The intra-assay variations as determined by the variation of the points on the standard curve for the LH-assay was 2.4% at 100% binding and 11.5% at 10-15% bound with a mid-slope value of 4.1%. For the FSH-assay the corresponding values were 2.1% at 100% bound, 13.5% at 10% bound and 3.7% at midslope. Synthetic LRH, a gift from Farbwerke Hoechst AG, Frankfurt, was injected via the indwelling catheter. The nasal spray, also a gift from Farbwerke Hoechst, was designed to deliver 100 µl containing 0.1, 0.5, 1.0 or 2 mg LRH in saline per application with an accuracy of about 10%.

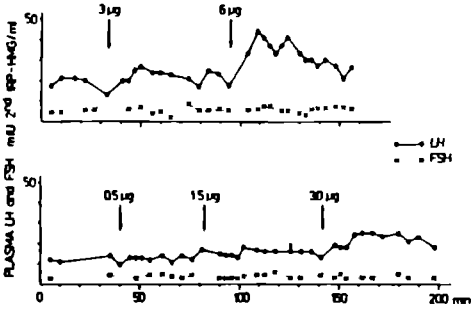


Figure 8.1: Continuous recording of plasma LH and FSH of two eugonadal female volunteers after intravenous injection of low doses of LRH.

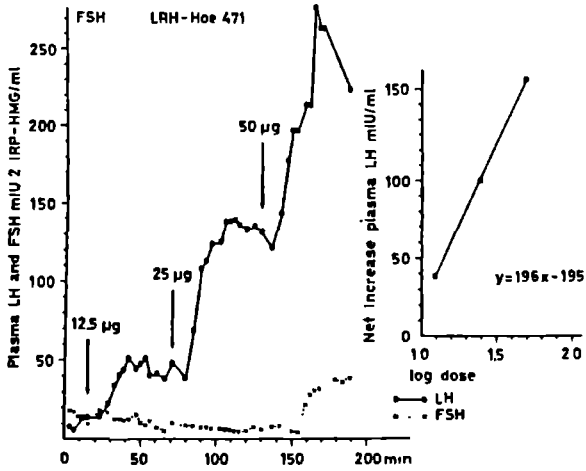


Figure 8.2: Effects of increasing doses of LRH on continuously recorded plasma LH and FSH levels of increasing doses of LRH in a eugonadal woman

## 8.3 Results

### 8.3.1 Response to intravenous LRH in eugonadal women

In order to detect small and short-lived changes we elected to establish the minimum effective dose and the time for maximum response by continuously monitoring the plasma LH and FSH levels.

LRH given at doses of 0.1, 0.2, 0.4, 0.5, 0.6 µg i.v. did not alter the basal plasma levels of LH and FSH. As seen in figure 8.1, 3.0 µg LRH was the minimum effective dose for the release of LH and FSH in two eugonadal female volunteers.Doses from 3 - 24 µg LRH resulted in LH release in the first 14 individuals tested. In 10 of these normogonadotrophic women FSH release was also noticed. Net increases in LH ranged from 9 to 110 mIU/ml, net increases in plasma FSH from 3 to 16 mIU/ml. These LRH doses caused the LH to reach a peak level after an average time interval of about 20 minutes (Table VIII.I).

Table VIII.I Time interval for LH peak levels following i.v. injection of low doses of LRH

| LRH dose (µg) | time interval min (range) | number of subjects |
|---|---|---|
| 3 | 18 (16-20) | 3 |
| 6 | 16 (14-20) | 4 |
| 12 | 21 (14-27) | 6 |
| 24 | 24 (10-31) | 10 |

In an attempt to induce a dose-dependent LH release LRH was injected in doses of 12.5 - 25 - 50 µg into a 26-year old eumenorrheal woman on day 9 of the menstrual cycle. As judged by laparoscopy, this individual had macroscopically normal ovaries. The time interval selected between the first and the second injection was 56 minutes and between the second and the third injection 59 minutes. THe net increases in plasma LH concentrations resulting from these 3 doses were 38, 100 and 156 mIU/ml respectively (fig 8.2). The time interval between injections did not allow circulating LH to return to resting levels. A linear relationship is evident when plotting LH increment versus log LRH dose. FSH concentrations were not altered after administration of 12.5 and 25 µg LRH, but injection of 50 µg LRH resulted in a 6 - 7 fold elevation of plasma FSH.

Dose-related FSH release was noted in a 40 year old eumenorreal subject when LRH was administered in doses of 6, 12 and 24 µg. Plasma FSH increments of 7, 11.5 and 17 mIU/ml were determined (fig 8.3). In a semilogaritmic plot of FSH increments versus LRH dose, a linear log dose response was found again. 8.3.2 Response to i.v. LRH in endocrinopathies.

Among 150 individuals tested with LRH in doses of 25 or 50 µg, 8 did not respond with a detectable gonadotrophin release. Figure 8.4 shows 3 typical examples. The upper recording was from a 22-year

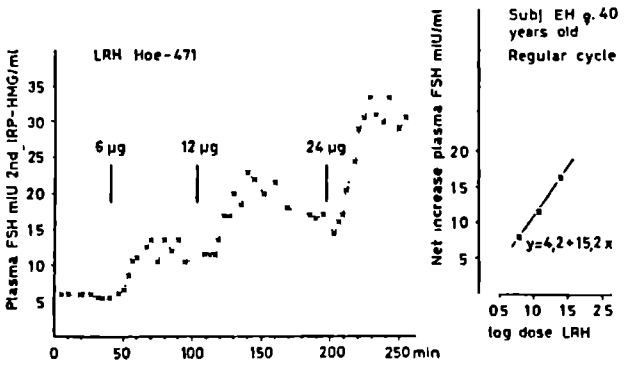


Figure 8.3 : Effect of 6, 12 and 24 µg LRH on continuously recorded plasma FSH in a eumenorrheal women.

old woman with a monophasic menstrual cycle and a history of no pregnancy after 4 years of marriage and continuing attempts at conception. Urinary ketosteroids were found to be subnormal at 3.6 mg/24 hours and urinary gonadotrophin activity was reduced to 10 IU/l. No reaction in plasma LH and FSH levels to 25 and 50 µg LRH was evident. The next recording shows the findings in a 15-year old girl with retardation of growth and puberty (height 146 cm, genital hypoplasia, infantilism, karyotype XX regular, normal plasma growth hormone levels). No reaction to repeated injections of 25 µg LRH was observed. The lower recording pertains to a patient with a typical Sheehan syndrome and a history of four pregnancies, hypothyroid with TBI of 1.48 and PBI of 1.7 µg/100ml, urinary ketosteroids 1.7 mg/24 hours. Here again, 25 µg LRH did not alter basal gonadotrophin levels. The inverted LH/FSH ratio in the last two cases should be noted.

We also found cases of over-responsiveness of the pituitary to LRH. In figure 8.5 4 cases are compared to a normal LH release pattern. In a 55 year old menopausal woman, basal LH concentrations ranging from 79 to 88 mIU/ml rose to a peak level of 210 mIU/ml 32 minutes after injection of the 25 µg test dose.

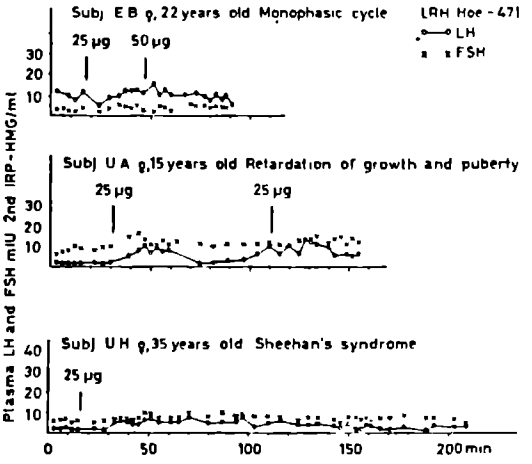


Figure 8.4 : Failure of synthetic LRH to induce LH and FSH release in three subjects with different endocrine disorders.

Similarly, in a peripubertal girl aged 9.5 years (bone age 12.5 years, menarche at 8.9 years, total urinary oestrogens 11.8-20& 6g/24h). The LRH responses showed a peak LH concentration of 326 mIU/ml after 27 minutes, starting from basal levels 4 - 6 mIU/ml.

Such overresposiveness was also apparent in hypogonadal and hypergonadotrophic men. For example, in a cryptorchid patient with aspermia and a subnormal urinary testosterone value of 6.3 µg/24h, the corresponding pattern was a basal LH level of 40 - 60 mIU/ml, rising to a peak of 223 mIU/ml after 19 minutes. Similarly, a patient with Klinefelter syndrome and basal LH levels of 42 - 48 mIU/ml exhibited a peak LH concentration of 147 mIU/ml after 21 minutes.

### 8.3.3 Response to intranasal LRH

The nasal spray was tested on five apparently healthy male volunteers, aged 21 - 37 years. They administered 0.1 - 2.0 mg LRH

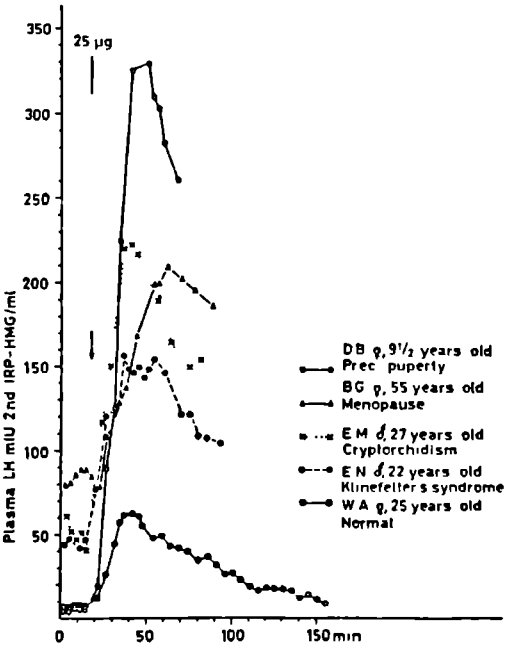


Figure 8.5 : Overresponsiveness of human pituitary to a test dose of 25 µg synthetic LRH as compared to normal reaction pattern.

to themselves. The average basal plasma LH value was 5.7 +/- 2.9 mIU/ml (mean +/- SD). This means that plasma LH values of up to 11.5 mIU/ml would have to be considered as falling within the normal variation (2SD). A spray of saline with no or with 0.1 mg LRH did not produce changes outside this normal variation. Application of 0.5 mg LRH led to detectable LH increases in all 5 cases. The average LH value 45 minutes after application of the spray was 13.9 +/- 7.4 mIU/ml and thus outside the above mentioned limit. We must therefore consider that a true LH release had in fact taken place in response to the intranasally sprayed LRH (figure 8.6). After administration of 1.0-and 2.0 mg LRH a readily apparent LH release was observed in all cases. For the 1 mg spray the average LH peaks was 18.4 +/-6.9 and for the 2 mg spray 30.2 +/- 7.3 mIU/ml. Basal FSH values were found to be 5.3 +/- 2.0 mIU/ml, which leads to a 2SD limit of 9.3 mIU/ml. The average FSH levels in this experiment remained below this limit (data not shown) and we must therefore conclude that even the highest LRH dose was insufficient to cause a clear FSH release.

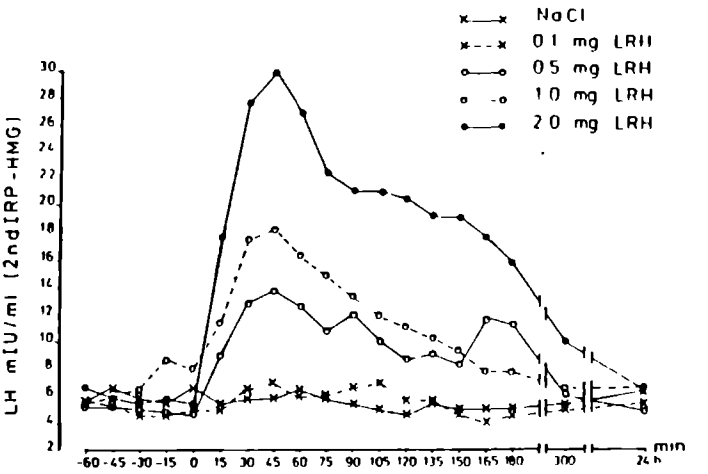


Figure 8.6 : LH release in normal men after intranasally sprayed LRH in doses from 0.1 to 2.0 mg (averages from five individuals).

Of the five female volunteers, three were given 0.5 mg LRH and two were given 2.0 mg on days 9, 10 and 11 of their menstrual cycles. In figure 8.7 it can be seen that after 0.5 mg LRH a significant LH-release is seen only on one day out of three in all subjects and that the net LH increase never exceeded 15 mIU/ml. In two cases the LH-release was seen on day 11 and in one on day 10. The administration of 2.0 mg to the remaining volunteers, however, resulted in a significant LH-release in both cases (figure 8.7). One of these women (K.G.) must have been close to the ovulatory LH-surge, as judged by the high basal values and high gonadotrophin response. Also, only in this case increased FSH release was noted.

### 8.3.4 Response to repeated intravenous LRH injections

Continuous monitoring of plasma hormone concentrations does not allow more than a few hours recording time in order to avoid excessive blood loss. Consequently, we always kept the recording time as short as possible. When using multiple LRH injections in

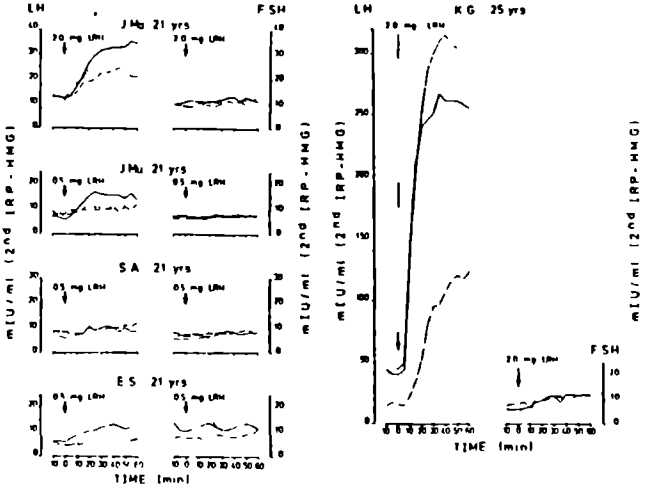


Figure 8.7 : LH- and FSH-release in normal females on days 9, 10 and 11 of the menstrual cycle after intranasally sprayed 0.5 mg or 2 mg LRH.

one recording session, this often led to the rather disturbing result that a dose-dependent LH-release could not be obtained. When however, the time interval between LRH injections was increased to about one hour, the expected LH dose-dependency could indeed be observed. It was also apparent that with repeated injections of the same LRH dose, the LH release was not reproducible at time intervals in the range of 30 - 40 minutes. Therefore, the time interval was considered to be of significance for the pituitary response and we set out to investigate this phenomenon further. Figure 8.8 depicts three typical unsuccessful attempts of inducing a dose-dependent LH release following intravenous injection of synthetic LRH in women. In the upper recording 6.25, 12.5 and 25 µg LRH gave rise to a rather disproportional LH release, when injected at 43 and 36 minute intervals in this 28-year old subject on day 26 of her regular menstrual cycle. The same pattern was observed in subjects with chronic anovulatory cycles, when larger LRH doses were chosen (12.5, 25 and 50 µg or 25, 50 and 100 µg LRH at 40 and 57 min. or 25 and 25 min. intervals in the two middle recordings). However, the administration of 12.5, 25 and 50 µg LRH at 56 and 60 minutes intervals respectively resulted in a dose-dependent response (the

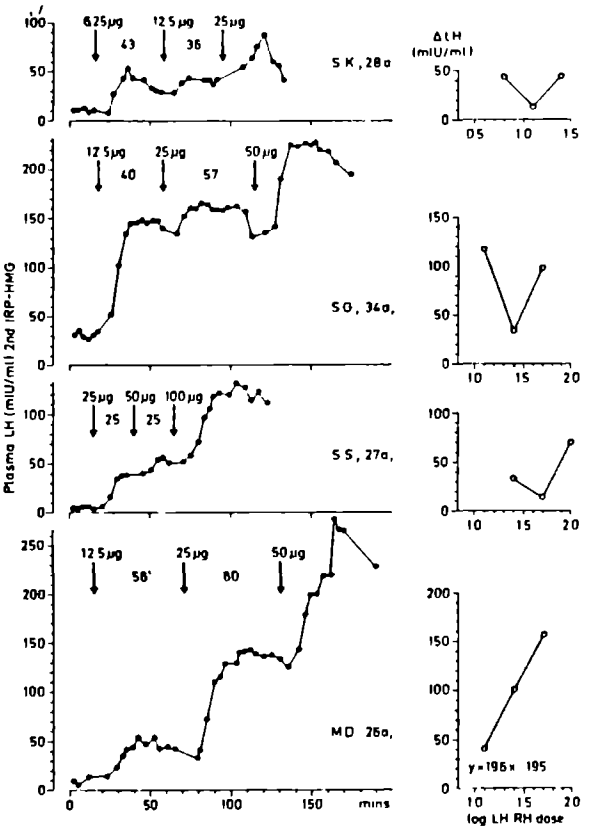


**Figure 8.8: Attempts to induce a dose dependent LH-release with synthetic LRH monitored by continuous recording of immunoprecipitable LH.**

lowest recording). When the net LH increments recorded in these four subjects are plotted against the logarithm of the LRH dose, linearity is only apparent in the lower recording.

In a group of seven patients the same LRH dose (12.5, 25 or 50 µg) was repeatedly injected at 30 minute intervals (figure 8.9). With one exception the second and third doses were less effective. This reduction in LH release was found to be significant at the 1% level for the second and at the 2% level for the third response.

In a different approach 25 µg LRH was injected at 30 min. intervals and this was repeated after a resting period of one

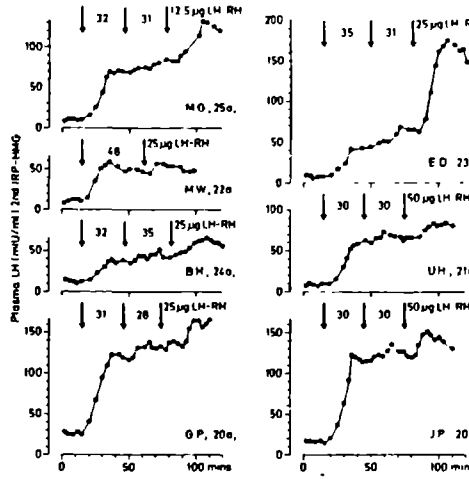


Figure 8.9: THe effect of repeated injections of the same dose LRH on immunoprecipitable LH in 7 subjects.

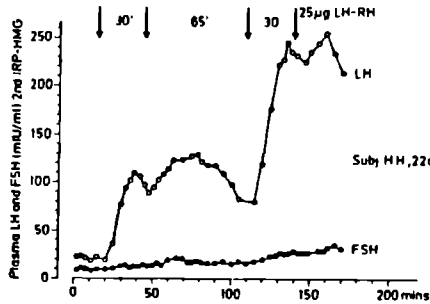


Figure 8.10: LH-release following two LRH injections 30 minutes apart, repeated once after a 65 minute resting period.

hour (figure 8.10). Again a typical LH release after the first injection was followed by a diminished response 30 minutes later. The one-hour interval allowed the LH-response to be fully restored, but 30 minutes later the fourth injection was as ineffective as the second injections. Net increments of LH in four subjects with anovulation, following two

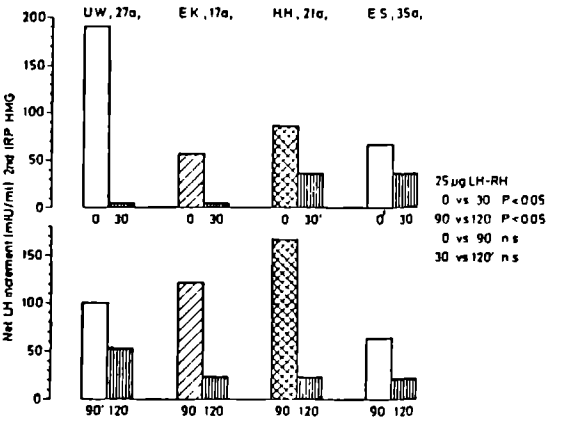


Figure 8.11: LH increments in 4 subjects following two pairs of LRH injections spaced 30 minutes with a one-hour resting period between the pairs.

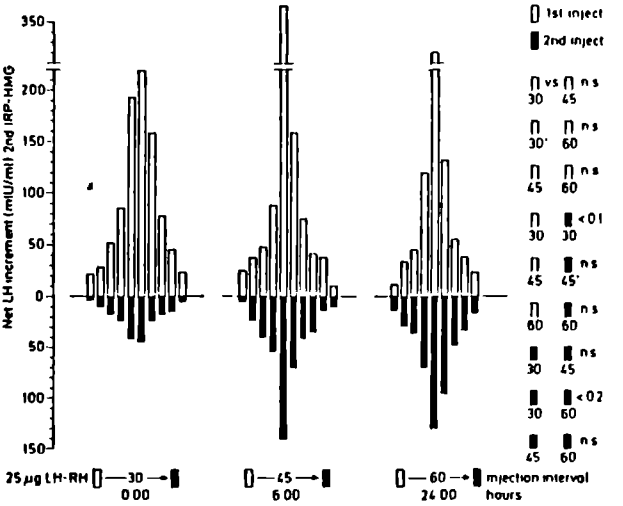


Figure 8.12: Net LH increments in response to paired LRH injections spaced 30, 45 and 60 minutes. The opposing open and solid bars represent the initial and subsequent LH increments resulting from the 25 µg LRH test dose.

pairs of 25 µg injections spaced as described above, are presented in figure 8.11. The maximum LH increase detected after the first releasing hormone stimulation (left bars) ranged from 55 - 191 mIU/ml, whereas the second response of the pairs (right bars) was lowered to a range of 3 - 35 mIU/ml ($p<0.05$). The corresponding results for the second paired stimuli 60 minutes later were 63 - 167 mIU/ml for the primary and 21 - 50 mIU/ml for the secondary respons ($p<0.05$). The primary as well as the secondary responses in the first versus the second pairs do not differ significantly.

Paired injections spaced at 30, 45 and 60 minutes were performed in the same subjects (female volunteers) at 0, +6 and +24 hours. Again, LH maxima were found by continouos recording of plasma LH concentrations. These 10 normal individuals at various days of the menstrual cycle revealed a considerable variation of the pituitary responsiveness to LRH. Nevertheless, a significant loss of responsiveness occurs when the paired injections followed within 30 minutes ($P<0.01$), but not at 45- and 60-minute intervals. When the initial or subsequent responses of the three treatment schedules were compared with each other (figure 8.12, opposing solid and open bars), a significant difference in the LH release pattern was evident only between the secondary injections in the experiments with 30- and 60-minute intervals ($P<0.02$). If the subsequent response is expressed in percent of the initial response, a significant decrease in LH release was detected after the 30-minute interval as compared to the 45-minute and 60-minute intervals approach ($P<0.01$ and $P<0.02$, respectively). No significant difference was detected between the responses to the 45- and 60-minute injection intervals.

## 8.4 Discussion

### 8.4.1 Initial studies

One would assume that the observed increases in plasma the LH and FSH levels would be due to an increased release of these anterior pituitary hormones. The possibility that their inactivation or removal is being blocked can be discounted on the basis of the following calculations. Assuming that the calculation of the daily removal rates for LH in the rat (Gay, Niswender & Midgley, 1970) also apply to man, we can assume that:

mIU/day = body weight in grams x mIU/ml plasma

In a 60 kg human with maximum LH levels of 20 mIU/ml the daily removal rate would be 1.20 million mIU/day or 833 mIU/minute. If

the blood volume is 1/13 of the body weight and the haematocrit is 45 %, then the total plasma volume is 2.07 litres. This results in a removal rate of 0.40 mIU/ml plasma per minute. With maximal responses occurring at 21 minutes after injection of LRH, a total block of LH degradation and removal from systemic circulation would account for an increase of no more then 8 mIU/ml. This rate of increase cannot account for the observed increases (40 - 120 mIU/ml).

Continuous recording of plasma hormone concentrations in man is limited by the total blood volume that can be withdrawn. Therefore, our experimental protocol did not allow for more then a few hours of recording time. Consequently, when several LRH injections were administered in one session, it was not possible to wait for the gonadotrophins to return to their baseline values. Nevertheless, linearity in the LH and FSH release was observed. This must be due to the relatively slow disappearance rate of gonadotrophins after LRH relative to normal plasma elimination. If the expected continuation of the gonadotrophin removal is extrapolated, linearity of the log-dose response still persists for both gonadotrophins (data not shown). A comprehensive study on this subject was done by Wollesen et al. (1976) who found a dose-response relationship with LRH doses between 1.5 and 3000 µg. This linearity seen in the release of LH and FSH suggests that synthetic LRH acts via a physiological mechanism and thus behaves like a natural hormone, as one would expect from natural human LRH. Furthermore, it supports the view of Schally et al. (1971) that LRH is a releasing hormone for FSH as well. However, in approx. 150 recording sessions conducted in 120 individuals, we did not regularly observe a significant FSH release.

### 8.4.2 The LRH test

One of the the aims of these studies was to obtain a better classification of certain infertility disorders in women on the basis of plasma LH and FSH levels and the results of LRH stimulation. Several publications were written in the seventies on the subject of the usefulness of LRH as a diagnostic tool to differentiate between different types of hypogonadism. After the initial euphoria of having a new hormone to work with, a number of investigatitors became rather disappointed when the use of LRH did not revolutionize diagnosis of infertility disorders. E.g. Mortimer et al. 1973, stated that the variation within groups is so large that gross characteristics of an individual's LH response is of little diagnostic value. It has also been reported that the response is related to basal values and does not distinguish between different pathologies and is

thus of questionable clinical value (Nilius & Wide, 1972; Mortimer et al. 1976; Coscia et al. 1974; Mortimer et al. 1973). It seemed to us that in order to obtain satisfactory differentiation, the methodology of the test would have to be standardized and expanded. This we attempted to do.

In our experience, 25 micrograms of synthetic LRH seems to be the most appropriate dose for evaluating pituitary function, as it gives rise to net LH increases ranging from 40 to 120 mIU/ml in eumenorrheal women. This increase parallels the physiological response of the anterior pituitary around the time of ovulation and leaves an assayable range for insufficient and overresponsive reactions to releasing hormone stimulation of the gland. Other investigators have suggested a dose of 100 µg (Mortimer et al. 1973, Patton et al. 1974; Taymor et al. 1974) but others favour lower doses in the order of a few tens of µgs. Schally et al. 1976; Crowley et al. 1985).

Our initial efforts (Schneider & Dahlen, 1972a, 1972b, 1973a, 1973b, 1973c, also reported here) led to a cooperative study at the Universities of Ulm, Tuebingen and Basel. This in turn led to the development of the so-called Human Pituitary Gonadotrophin Index (Keller et al. 1976), which is based on the statistical evaluation of 339 carefully standardized LRH tests with 25 µg LRH and a sampling of LH and FSH before and after stimulation. The index classifies patients into several categories according to basal LH levels and LH responses. Combination of this index with a clomiphene test (clomiphene is thought to be acting on the hypothalamus) and an investigation of the pulsatile LH patterns (Bohnet et al. 1975), may be provide a useful evaluation of patients before, during and after therapy for fertility disturbances (Bohnet et al. 1976).

Although LRH has now been available to clinicians for several years, there are still publications appearing, which report new results from using the LRH test in the diagnosis of hypothalamic - pituitary - gonadal dysfunctions. It has been reported that the relationship between basal LH and LRH response differs in men with or without hypothalamic - pituitary dysfunction and this phenomenon can be used to improve the diagnosis of pituitary hypogonadism (Harman et al. 1982). In elderly men there seems to be a delayed response (Winters & Troen, 1982; Harman et al. 1982) and a diminished response in diabetic patients with amenorrhea (Djursing et al. 1983).

8.4.3 Abnormal responses

In the first 150 LRH tests we observed eight instances without detectable increase in plasma LH concentrations. Such lack of pituitary responsiveness to synthetic LRH could be due to either primary or secondary pituitary failure. Primary failure is commonly seen in patients with pituitary necrosis, tumour, trauma or empty sella syndrome. However, it must be born in mind that pituitary function may remain normal even when large amounts of pituitary parenchyma have been destroyed (van Buren & Bergenstal, 1960). This type of pituitary failure is illustrated in the present study by the recording from a patient presenting a typical Sheehan syndrome. The low but still detectable LH and FSH levels should be noted. As pointed out by Ross (1972), the mere presence in plasma of immunoassayable gonadotrophins does not necessarily imply continuation of their trophic function. Maqueo et al (1972) demonstrated a lack of antral follicles in 92% of a large group of women using oral contraceptives, although this treatment is known to leave seemingly sufficient plasma gonadotrophins. The other two instances of no response, demonstrated here, represent a group of patients with clinically suspected secondary pituitary failure i.e. with a lack of proper endogenous LRH stimulation, resulting in insufficient gonadotrophin release, which is also noticable after exogenous LRH administration.

In contrast to an insufficient response, the other extreme, an overresponsiveness, is regularly observed in patients presenting such endocrine states as menopause, castration, cryptorchidism, Klinefelter's syndrome and onset of puberty. All these endocrine disorders have in common a hypogonadal and hypergonadotrophic status and thus an open-loop condition in terms of gonadal feedback control. The open-loop is associated with high endogenous LRH activity, as suggested by the results of in vivo studies in basic reproductive endocrinology. In the primate and human menstrual cycles, the highest sensitivity to LRH is found during mid-cycle (Knobil, 1974; Saito et al. 1972; Crowley et al. 1985).

The chief criticism of the LRH test has been that it does not always distinguish between hypothalamic and pituitary causes of amenorrhea. A normal response to LRH does not always indicate hypothalamic dysfunction, since even a diseased pituitary can release LH (Schally et al. 1976) and the absence of a response does not always indicate a pituitary disease. A normal pituitary deprived of (prolonged) LRH stimulation caused by a hypothalamic lesion may lose its capacity to respond. Patients with anorexia nervosa show a poor response to LRH, although the cause of the problem undoubtedly originates above the pituitary (Van der Kerckhoeve, Dhont & van Eyck, 1975; Nillius & Wide, 1972; Schneider & Dahlen, 1973a). It is

also important to note in this context that anovulation could also be caused by dysfuntion of a brain centre responsible for cyclical functions. Not only must LRH signals be present to induce LH release, they must also take place at the right time and with the right frequency in order to lead to a proper ovulatory cycle (Crowley et al. 1985). Such considerations lead to the conclusion that the LRH test alone does not suffice in all cases and must be supplemented by other tests. A clomiphene test can give useful information about the functional capability of the hypothalamus (Bohnet et al. 1976). Estimates of endogenous LRH release can be obtained by measuring the pulsatile LH release patterns (Bohnet et al. 1975; Crowley et al. 1985). State of the art diagnosis of hypogonadism today is employing a combination of LH pulse recording, either in response to endogenous or exogenous LRH in a pulsatile manner, and in addition is the response recorded to LRH replacement therapy based on physiological doses and time intervals. (Crowley et al. 1985).

Our data support the concept that pituitary sensitivity to exogenous LRH depends on the degree of endogenous gonadotrophin releasing hormone stimulation. Thus, varying degrees of gonadotrophin release to a standard stimulus can occur. The extreme sensitivity to LRH seen in a girl at the onset of puberty (figure 8.5) can also be explained in terms of high levels of endogenous releasing hormone. Animal experiments indicate that this phenomenon can be explained by the fact that LRH can induce its own receptor. Pituitary LRH-receptors increases late in dioestrous during the rat oestrous cycle and remain high until late pro-oestrous (Clayton et al. 1980). Furthermore, low doses of LRH can increase receptor the content in rats and the treatment also leads to increased LH responses (Frager et al. 1981). Gonadectomy has also been shown to increase the receptor levels (Marion, Cooper & Conn, 1981; Clayton & Catt, 1980). This can be interpreted to mean that the rate of hypothalamic LRH secretion will determine the number of pituitary receptors, which then provides a means of modifying gonadotrophin secretion.

### 8.4.4 Intranasal LRH

The results from the nasal spray experiments confirm that synthetic LRH is indeed capable of releasing LH when applied intranasally. A significant LH-release is seen only when doses of 0.5 mg or more are used. The release after 2 mg is in the same range as seen after i.v. injection of 25 µg LRH, indicating that about 1 % of the applied LRH reaches the circulatory system in a biologically active state and can thus act on the pituitary. Studies with a LRH-radioimmunoassay suggest that indeed about 1 % of the intranasally

applied LRH is detected in the plasma (P. Franchimont, personal communication), which suggests that there is a considerable intranasal loss of LRH.

The time-course for the LH-release reveals a retarded release (peak at 45-60 minutes) compared to the response after i.v. injection (peak at 20-25 minutes). This was to be expected as the LRH must first reach the circulatory system in appreciable quantities before it can act on the pituitary. This retardation in the maximum response is probably due to slow absorption of LRH. London et al. (1973) have reported peak LH-values at 30 minutes rather then the 45 minutes reported by us. The discrepancy may be due to the fact that these authors did not include sampling at 45 minutes but only at 30 and 60 minutes and so they may have missed the true peak.

Although this mode of administration employs relatively large amounts of LRH, this disadvantage is more then outweighed by the ease of application. The fact that little or no FSH is released may limit the use of the spray method. Nevertheless, the chronic mode of LRH therapy has more aspects than gonadotrophin release alone. E.g. the anovulatory female, not responding to clomiphene due to hypothalamic dysfunction, can be converted into a clomiphene responder by chronic LRH therapy (Bohnet et al. 1976). In the rodent this kind of treatment exerts a stimulatory effect on endogenous releasing hormone activity, when subcutaneous injections are used (Sandow & Heptner, 1974). Furthermore, in monkeys with lesions in the hypothalamus, continuous infusion of the releasing hormone did not support the gonadotrophin secretion. After initial increases the pituitary became refractory and the levels fell. However, the administration of LRH in a pulsatile manner once per hour resulted in a normal secretory pattern of the gonadotrophins (Knobil, 1980 & 1981). This mode of application was sucessful in the induction of ovulation in women with amenorrhea (Leyendecker, Struve & Plotz, 1980). The LRH spray is ideal for this kind of replacement therapy as has been shown by several investigators (Hagberg & Westphal, 1982, Cacciari, Frejaville & Becaa, 1982). The opposite effect, inhibition of ovulation, can also be induced by means of the LRH spray (Bergquist, Nillius & Wide, 1979 & 1982). This, initially rather surprising, contraceptive effect might depend on overstimulation and a resulting down-regulation of pituitary and/or ovarian receptors.

### 8.4.5 Refractoriness of the pituitary

Our studies with different time intervals for sequential paired LRH injections present evidence for the existence of a partial refractory period of the pituitary gonadotroph amounting to least 30

minutes. It is also evident that these phenomena are independent of a possible diurnal rhythm, since the response pattern remains unchanged when the experiments were carried out at different times of the day. Furthermore, there are no changes in the refractory pattern during the various stages of the menstrual or an anovulatory cycle.

A look at the relative LH responses reveals that the second response is on the average about 30, 60 and 75 % of the initial one after intervals of 30, 45 and 60 minutes respectively. In other words there is a gradually diminishing response by the pituitary lasting for about one hour. Under physiological conditions the maximal frequency for the pulsatile pattern of basal LH release is also about one hour (Yen et al. 1972b). Thus, it could be that the pituitary gonadotroph responds with a similar refractory pattern to endogenous LRH stimulation and does not allow for unlimited transmission of the neurohormonal stimulus.

Similar results were obtained by Crowley et al. (1985) when they investigated the effects of changes in frequency of LRH administration to hypogonadal men. When gradually increasing the frequency from a pulse every 2 hours to one every 15 minutes, there was a progressive loss of response and they noted non-transmitted pulses of exogenous LRH. Throughout the experiments the serum testosterone level remained unchanged.

An explanation for this phenomenom can be found in modulation of the numbers of cell surface receptors in response to hormone concentration. As discussed above in section 8.4.2 LRH can induce its own receptors. However, there is another aspect to LRH - receptor interactions. Once LRH is bound to the receptor, a sequence of events starts, leading to a disappearance of the receptor from the cell surface. After 20 minutes the LRH-receptor complex forms clusters, which after 30 minutes are internalized (Hazum et al. 1980). This means that there is a transient loss of receptors available on the cell surface. Working with cultured rat anterior pituitary cells, Loumayer & Catt (1982) could show an initial loss of receptors, which reaches a maximum after 1 hour. This down-regulation was followed by a recovery and sub- sequently an increased number of receptors.

These findings may be relevant for clinical pituitary function tests. A negative result would suggest either pituitary insufficiency or hypothalamic failure. However, the possibility that a normally integrated hypothalamo-pituitary system was stimulated during a refractory period must be eliminated by repeating the test at a later time.

There are also obvious consequences for the chronic use of LRH e.g. to stimulate secondary gonadal immaturity. For a number of years chronic LRH therapy was not giving the expected results. Infusions failed to sustain gonadotrophin secretion for reasonable periods of time and "the pituitary appeared to become refractory to LRH" (Knobil, 1980). Apparently these authors did not notice our studies of repeated LRH administrations (Schneider & Dahlen, 1973b and 1975), as they make no reference to them. Working with rhesus monkeys, Knobil then proceeded to administer the LRH in pulses. While 5 pulses per hour extinguished gonadotrophin secretion, one pulse per hour gave the desired gonadotrophin increases.

## 8.5 Summary

By means of a system for continuous monitoring of LH and FSH in women we established the lowest effective dose of LRH for causing a release of LH and FSH (3 - 6 (g). Dose-related responses could be induced in the release patterns of both gonadotrophins. The use of the releasing hormone as a diagnostic tool was investigated by measuring the responses to LRH stimulation in women with a variety of endocrinological disorders. Over-responsivenes as well as insufficient responses were noted in conditions with elevated and decreased endogenous activity, respectively. A nasal spray mode of administration for LRH was investigated and the effectiveness of the method checked. During the determination of dose-response curves for LRH in man, it was often impossible to achieve linear responses. By means of sequential injections spaced at different time intervals, this could be shown to be an effect of pituitary refractoriness to releasing hormone stimulation. The implications of this effect are discussed.

# CHAPTER 9

## General discussion

Elucidation of the important roles played by peptides in the central regulation of various endocrinological functions has led to the discovery and isolation of a number of hypothalamic hormones that regulate secretions of the anterior pituitary gland. The list of neuropeptides in the hypothalamus is ever increasing. Summing up evidence from radioimmunoassay and immunocytological experiments, Palkowitz (1982) cited evidence for 14 neurologically active peptides in the rat median eminence, at least 5 with effects on LRH secreting neurons. It seems that the more we learn about hypothalamic processes, the more complicated is the overall picture. In this report we try to give an account of our efforts to improve the understanding of certain aspects of reproductive neuroendocrinology.

### 9.1 Radioimmunoassay of LRH

The availability of synthetic LRH opened up new areas to investigations into the mechanisms of reproduction. One of the new possibilities was the development of radioimmunoassays for the decapeptide to enable, for the first time, its precise measurement in biological fluids and tissues. This technique combines great simplicity and high sensitivity with the specificity inherent in immunological reactions. However, radioimmunoassays have their special problems, which require particular care in their development and execution. It has been recommended by the World Health Organization (1972) that in determining antiserum specificity the investigator should compare the results with an established bioassay and that inhibition curves for unknowns must be parallel to the standard curves.

Ideally, the comparison should be made in the medium in which the radioimmunoassay is going to be carried out. This does not always produce a true picture of the concentration in the biological fluid. In plasma for instance, factors such as enzymes and other compounds are frequently interfering. This can take place at different levels. First, the compound to be measured may be metabolized during storage or assay of the sample. Oxytocin for instance, depends on an intact molecule, and in particular on the integrity of its ring structure. The breakdown in the presence of oxytocinase, e.g. in pregnancy plasma, is much more rapid when measured by bioassay than as seen in a radioimmunoassay (Chard et al. 1970a). When in-

fusing the hormone, the estimates by the two methods show more difference as time passes (Forsling, Boyd & Chard, 1971) Secondly, the assay may be influenced by some factors present in the sample This is particularly true for the radioimmunoassay of LRH in plasma (see Chapter 7) These problems can often be eliminated by immediate deproteinizing of the sample followed by an extraction procedure Consequently, we decided to include these procedures This enabled us to compare the biological and the immunological assays in the extraction medium In our studies reported here, both criteria suggested by the WHO for radioimmunoassay validation could be met comparison with the Ramirez-McCann bioassay, utilizing oestrogen-progesterone primed ovariectomized rats, showed excellent agreement and hypothalamic extracts and synthetic LRH showed parallel inhibition curves

These are essential elements of radioimmunoassay validation but, unfortunately, not enough The possibility that metabolic products or precursors may be detected must also be excluded Such compounds will contain elements of the structure of the original structure to varying degrees and it is thus conceivable that the antibodies will not distinguish between them and the substance itself In our studies we could show that our antisera were highly specific but that an anti- serum supplied by other investigators showed a lack of complete specificity in that it was specific for the N-terminal of the decapeptide only

This has more than theoretical consequences as shown by the studies of Jeffcoate et al (1973b and 1974a) utilizing this antiserum for determinations of LRH in urine. The studies, employing gel chromatography and thin layer chromatography in addition to the radioimmunoassay, suggested that the releasing hormone was present intact in urine Hence, urinary measurements of LRH would be expected to yield physiologically meaningful results We could, for instance, follow the 24-hour production pattern and look for increases e g at times of elevated plasma LH levels However, subsequent studies employing carboxymethyl-cellulose chromatography (Jeffcoate & Holland, 1975), showed that the immunoreactive entity was in fact the 3 - 10 octapeptide metabolite of LRH. This evidence was further confirmed by studies on the metabolic fate of LRH by Redding & Schally (1972) and by Redding et al. (1973) They could show that LRH is partly broken down by splitting off of the dipeptide pyroglutamyl-histidine, which lead to the formation of the above mentioned octapeptide.

Similarly, Aubert et al. (1977) and Copeland et al (1976) using three antisera, obtained only minor differences when assaying LRH in a human foetal hypothalamic specimen (1.5, 1 6 and 1 8 ng respectively) whereas a urinary sample yielded widely differing results (0 7, 3.5

and 7 ng respectively). In other words, when assaying hypothalamic samples, where we can expect less degradation products than in urine, the choice of antiserum is not so critical. However, for assays in urine it is of crucial importance.

The biological activity of LRH is lost when substitutions are made in the four N-terminal amino acids (Yanaihara et al. 1973a and 1973b), indicating that groups essential for the biological activity reside in this part of the molecule. This means that antisera specific for the N-terminal only, might show better correlation with bioassays than an antiserum specific for the C-terminal only. On the other hand, the latter antiserum only responds to peptides with the intact, metabolically sensitive C-terminal which would make it insensitive to the metabolic breakdown products. It is obvious from the above that an antiserum, such as produced in this study, that requires both ends of the LRH molecule for binding, provides a very high degree of specificity and a large probability of correlation with bioassays.

Another metabolic route for LRH was described by Marks & Stern (1974) who could show that, in addition to the previosly mentioned C-terminal breakdown, there are also neutral endoproteases capable of internal cleavage of the decapeptide. This will produce C- as well as N-terminal fragments. As however the antisera used in this study required both terminals not only to be present but also to be attached to each other (Table V.1), the breakdown products of this pathway will not be detected by our LRH radioimmunoassay either.

The only way that metabolic events could interfere with the results from our radioimmunoassay would be by a rapid breakdown of LRH in the biological environment, by binding of the releasing hormone or by influencing the reaction between LRH and the antibodies. Such interference was noted by us and by others. The rapid breakdown could be largely circumvented by immediately deproteinizing the samples by the addition of cold ethanol. This treatment also initiates the extraction procedure which also removes compounds interfering with the radioimmunoassay and in addition concentrates the samples and thus increases the sensitivity.

### 9.2 Immunoreactive LRH levels in peripheral plasma

When searching the literature for "normal values" for LRH in plasma measured by radioimmunoassay we find a wide range of estimates (Table IX.1)

Table IX.1 Immunoreactive LRH in plasma

| Investigators | species | concentration (pg/ml) |
|---|---|---|
| Kerdelhue et al. (1973a and 1973b) | sheep | 6000 - 12000 |
| | rats | 4000 - 5000 |
| Keye et al. (1973) | men | 68 +/- 17 |
| | women | 70 +/- 12 |
| Crighton et al. (1973) | sheep | <10 - >10000 |
| Fraser et al. (1973) | rats | <10 - 900 |
| Nett et al. (1974) | sheep | 120 - 370 |
| Arimura et al. (1974) | women | 1 - 17 |
| Jeffcoate et al. (1974a) | sheep | 10 - 230 |
| | women | 0.25 - 9.5 |
| Rosenblum et al. (1975) | men | <5 - 40 |
| Jonas et al. (1975) | sheep | n.d. - 400 |
| Foster et al. (1976) | sheep | 30 - 220 |
| Aksel et al. (1979) | men | 20 +/- 5 |

n.d.= not detectable, range or mean with SEM given

Although Table IX.I does not present a complete list of all publications on this subject, it serves to indicate the wide range of LRH levels in plasma that have been reported. The lower estimates are far more likely to have any physiological meaning. In chapter 8 we could show (Schneider & Dahlen 1972b) that the lowest dose of exogenous LRH that is capable of releasing LH was approximately 5 (g. In an average person with a plasma volume of 2 litres, assuming that the LRH is distributed only in this compartment during the first minutes after intravenous injection, this would mean that the minimum effective concentration is approximately 2.5 ng/ml. Endogenous LRH, however, is secreted into the portal vessels, which leads to an approximately 500-fold dilution before it enters the general circulation. This means that we can assume that a minimum effective LRH secretion from the hypothalamus will lead to a LRH concentration in the general circulation of approximately 5 pg/ml. A dose of approx. 100 (g exogenous LRH causes maximum response. If the hypothalmus is capable of providing such maximal stimulation, plasma LRH values of up to 100 - 200 pg/ml would represent the upper limit of the physiological range.

The results of Clemens et al. (1975) support the validity of the above calculations. They measured 200 pg/ml circulating after injection of 1 (g LRH / squaremetre body surface, a dose which they had determined to be the minimum effective dose. Recalculated to match our conditions, this would lead to a value of 1 pg/ml in general circulation resulting from a minimal hypothalamic LRH secretion capable of stimulating LH release. The minor difference as compared to our calculated estimate (5 pg/ml) can probably be neglected.

Malacara, Seyler & Reichlin 1972, using a sensitive bioassay and an extraction method showed that small amounts of circulating biologically active LRH is present in peripheral plasma of women and that increased amounts were present during mid-cycle. Approximate calculations from their results suggest that concentrations of 25 pg/ml might be expected at that time.

Considering the above, we must doubt reports with plasma LRH values in excess of 1000 pg/ml, such as some of those cited in Table IX.1. It is difficult to accept values above a few hundred pg/ml as having any real physiological significance. This is especially so in the cases where the assay was performed on unextracted plasma. In our studies the values are within the limits calculated on the basis of the results with exogenous LRH.

In view of the high specificity of the radioimmunoassays and the precautions taken to eliminate interference, we consider that the levels of immunoreactive LRH in peripheral plasma extracts, observed by us and certain other investigators, are very unlikely artifacts and probably represent the presence of the decapeptide known as LRH. A different matter is the origin of the peptide. In the light of recent findings of extra-hypothalamic LRH in various organs, we must now consider that perhaps only a (small?) part of the detected LRH is of hypothalamic origin. It seem more likely that we are dealing with LRH from a number of sources. The physiological functions of LRH outside the hypothalamic - pituitary region are largely unknown and speculative.

### 9.3 LRH content of the hypothalamus

Although we did not perform any detailed anatomical studies, the distribution of LRH in the brain we have found is in agreement with that reported by others. LRH is concentrated in the medial basal hypothalamus, lesser amounts are found in the anterior hypothalamus (McCann, 1962; Crighton, Schneider & McCann, 1970; Wheaton, Krulich & McCann, 1975). This distribution may be interpreted in terms of our present understanding of the hypothalamic control of gonadotrophin secretion. It is generally assumed that two centres are involved in this process. One centre, located in the preoptic nucleus, is thought to be responsible for cyclic LH functions such as the ovulatory surge. A second centre, including the arcuate nucleus, is thought to be involved with the tonic secretion of LH, i.e. the maintenance of basal LH levels. We consider that LRH is synthesized in the neuron perikarya in the suprachiasmatic - preoptic area and is transported via long axons down to the median eminence, from where it is released into the portal vessels. Another set of neurons, which

have their cell bodies in the arcuate nucleus, is also thought to produce LRH and to deliver it via shorter axons to the median eminence (Schneider & McCann, 1969; Zimmerman et al. (1974, Krulich et al. 1977b; Paden & Silverman, 1979; King et al. 1980; Jenner & Stumpf, 1980; Witkin, Paden & Silverman, 1983).

In our studies on the effect of removing gonadal steroid feedback we have limited the measurements to the median eminence and surrounding structures. About 90 % of the total hypothalamic LRH is found in this region (Wheaton, Krulich & McCann, 1975). In view of the concept cited above, this is not very surprising. Furthermore, Ramirez et al. (1975) could show that the vast majority of hypothalamic LRH is contained in nerve endings, i.e. the median eminence. In other words, as regards hypothalamic LRH content, it is permissible to substitute the retrochiasmatic hypothalamus used in this study.

### 9.4 Role of biogenic amines in LRH secretion

The activity of the neurosecretory LRH neurons is influenced by a large number of putative neurotransmitters capable of either stimulating or inhibiting the release of LRH (McCann, 1982, Barraclough, Wise & Selmanoff, 1984). It has especially been suggested that dopamine and norepinephrine are involved. McCann, 1969; Gnodde & Schuiling, 1976; Kizer et al. 1976; Vijayan & McCann, 1978a; Rance et al. 1981; Ojeda et al. 1982. Although significant advances have been made in this field, e.g. the involvement of endogenous opioid peptides (Ferin et al. 1984), the precise role of the various catecholamines are still largely unknown. The questions as to whether there is a stimulatory or inhibitory role of dopamine and norepinephrine in LRH release and whether dopamine or norepinephrine (or both) is involved in the triggering the release of LRH to induce the basal (LH-episodes) and cyclical (ovulatory LH-surge) has yet to be clarified. There are evidence that modifications in the steroid enviroment could lead to the different effects and thus might explain the contradictive results that have been obtained in the past (McCann, 1983a).

Just about the only thing that we can say with absolute certainty on this subject is that the control mechanisms involved with the hypothalamic releasing hormones are very complex. Simple incubations and perifusions such as in our studies are not likely to yield meaningful information unless far more attention is given to factors such as choice of tissue and steroid environment. Recently Drouva et al. (1984) could show that the hypothalamus from ovariectomized rats releases less LRH (in vitro and in response to depolarization with potassium) than that from rats receiving oestrogen replacement. The

studies of Leadem & Kalra (1984) showed that the median eminence from pro-oestrus rats can release LRH into perifusate when stimulated with progesterone. Similarly Kim & Ramirez (1985) could show LRH release from the hypothalamus of oestrogen primed rats in response to steroid treatment in vitro. These studies and others demonstrate the importance of having a carefully defined model, especially in terms of steroids, for in vitro experiment involving LRH release.

This is also exemplified by the fact that when the median eminence is attached to the rest of the hypothalamus, progesterone can only stimulate LRH release (in vitro) when administered in pulses whereas the median eminence alone will respond also to a continuous exposure (Ramirez et al. 1985). In addition, this is another example of the effect shown in the experiments by Negro-Vilar et al. (1979) and Gambacciani et al. (1986) where the whole hypothalamus was much less responsive than the median eminence. Thus, the use of hypothalamic fragments, slices, cultures or homogenates may give rise to false negative data as well as artefactual positive data, depending on the nature of the missing function. On the other hand, for some type of experiments we may have to employ precisely such a fragment. In our studies we seem to have been making the wrong choice of tissue (intact hypothalami).

Other factors might also have influenced the results and gave rise to the poor stimulation results. Investigations by Bennet et al. (1975) showed that when preparing synaptosomes from hypothalamic tissue, dopamine could evoke a LRH response from only 1/10 of the cells. Thus, with 90 % of the hypothalamic cells unresponsive it is easy to see how contradictory results can be be obtained. Furthermore, we should remember that we are dealing with a rather artificial situation when doing in vitro experiments. This is exemplified by the apparent loss of viability of hypothalamic tissue seen in figure 7.5 where large amounts of LRH is released into the medium, presumably due to tissue decay. We can also question if the perifusion system really provide for an equal distribution throughout the tissue for both life sustaining nutrients and essential factors as well as putative stimulants.

The importance of having the right steroid enviroment was also recently demonstrated in vivo by Peduto & Makesh (1985). They, in analogy to one of our experiments, attempted to induce LRH release by means of progesterone in oestrogen primed ovariectomized rats. As in our experiments were levels of immunoreactive LRH in peripheral plasma not changed with the dose levels used by us. However, they were able to show that our basic concept was fundamentally sound. A reduction of the amount of steroids to more physiological levels did indeed bring about significant LRH increases. In other words, the

doses of the steroids for the priming are of crucial importance in order to show the progesterone effect.

### 9.5 Clinical studies

The nasal spray tested in our study had been developed by Hoechst AG for use in conditions of endogenous LRH insufficiency, leading to disorders of reproductive functions, e.g. azoospermia, failure of ovulation and delayed puberty. Our discovery, confirmed by others, that the pituitary is desensitized in response to repeated or continuous administration of the releasing hormone led to a rather surprising development, namely that such sprays have been used for anti-conception purposes (Bergquist, Nillius & Wide, 1979 & 1982), i.e. they are used to inhibit ovulatory processes rather than induce them. LRH-analogues in the same spray as we used in the trial for Hoechst are also being used for treatment of sex-hormone dependent carcinomas (Klijn & de Jong, 1982; Tolis et al. 1982) as well as endometriosis (Meldrum et al. 1982). However, used in a proper mode, i.e. pulsatile, the LRH spray can also be used for replacement therapy (Hagberg & Westphal, 1982; Cacciari et al. 1982).

It turned out that the LRH test alone does not allow for a complete evaluation of the hypothalamic - pituitary system in the course of diagnosing reproductive dysfunctions. As a result was the estimation of the pulsatile LH release pattern introduced to assist. Both approaches, monitoring either the results of endogenous LRH secretion or the results of exogenous LRH, have inherent limitations and a combination of both is needed to maximize information about the dysregulated gonadostat. As seen in our studies, the LRH test might turn out negative as a result of hypothalamic malfunction resulting in an LRH stimulation insufficient to maintain the proper receptor numbers in the pituitary and hence no LH release upon LRH administration. A possible cause for a false negative result would be that the anterior pituitary becomes refractory also to an endogenous pulse of LRH and hence will not react to the endogenous releasing hormone. When monitoring pulsatile LH patterns in order to gain an insight of patterns of LRH secretion from the hypothalamus, one assumes that the pituitary responds to every LRH pulse with a corresponding LH release. This is not necessarily so, the refractory period demonstrated by us could lead to a blocked or non-transmitted pulse.

It is quite possible that a dual mechanism exists for the regulation of LH secretion. This could mean that a pulsatile releasing hormone pattern is superimposed on a refractory periodicity of the pituitary. The results of Clarke & Cunnings (1982) and Levine et al. (1982) point in this direction. These investigators could show that

LRH pulses are present in the sheep portal system without corresponding LH episodes in the general circulation, but that LH release is never found without a LRH pulse. Although these results could be due to experimental artifacts, we have to seriously consider that the LRH signals are modified at the pituitary level. Factors that might be involved in the regulation of such effects are, obviously, steroids and catecholamines. Receptors for such compounds have been found in the anterior pituitary (Kato & Onouchi, 1977; Weiner & Ganong, 1978). Concentration of LRH receptors on the membranes of pituitary cells has also been seen to vary with steroid environment (Crowley et al. 1985; Clayton & Catt, 1980; Moss, Crowder & Nett, 1981; Adams, Norman & Spies, 1981). Another obvious candidate for such effects is LRH. It is now clear that LRH is influencing the pituitary receptor levels. As we have seen this can be a down-regulation in response to repeated or continuous exposure. Conversely the pituitary may be primed by small doses in a pulsatile manner which will lead to an increased number of receptors. This way can hypothalamic centres influence the pituitary, not only by a pulse to induce an LH episode, but also to induce changes in sensitivity to the LRH pulse.

# CHAPTER 10

## Summary

### 10.1 English

A radioimmunoassay for Luteinizing Hormone Releasing Hormone (LRH) has been been established, tested and applied. A method for the preparation of antibody to LRH has been worked out (chapter 3). This consists of administring LRH coupled to bovine serum albumin to rabbits by means of an intradermal multiple site injection tech- nique. For the labeled antigen we choose radioiodinated LRH, which could be prepared by the Chloramine T method. A subsequent Sephadex gel-chromatographic purification step yielded a suitable tracer for the radioimmunoassay of LRH.

Optimal conditions for the performance of the LRH radio-immunoassay with regards to incubation time, incubation temperature, concentration of antiserum and radiolabeled LRH have been established (chapter 4). The affinity constants of LRH for the antibody were determined in antisera from a number of bleedings. Two different methods for the separation of bound and free antigen were examined, the double antibody technique and solvent precipitation of the antibody (chapter 4). The latter technique was chosen for routine use. The preparation of standard curves is discussed in detail. A phase separation method was chosen and the type of incubation schedule with the best performance was selected. Three different antisera were compared and the precision of the assay method was determined.

The specificity of the LRH-radioimmunoassay was also investigated (chapter 5). The immunological specificity was established by measuring the ability of LRH-analogues to displace radiolabeled LRH from its binding to the antibodies. Of the three antisera checked, two were found to be highly specific in that they required both ends of the LRH molecule for binding, while the third was found to be specific for the C-terminal only. The high specificity was maintained for a prolonged period of time during production of antiserum. In order to check the biological specificity a bioassay for LRH was established. This required in turn the setting up of a radioimmuno-assay for rat LH. The results obtained show excellent agreement between the two different methods. Further validation of the LRH radio-immunoassay followed from the parallel inhibition curves for hypothalamic extracts and synthetic LRH, reported in chapter 7.

Direct measurement of LRH in plasma by means of radioimmuno-assay was found to have many pitfalls (chapter 6). Proteolytic en-

zymes can cause degradation of LRH, and other (macromolecular) plasma components can interfere with the assays. This non-specific interference can largely be eliminated by the extraction procedure, which we have developed and validated. Continuous sampling is essential when measuring LRH in the circulation, as the releasing hormone seems to be secreted in a pulsatile manner and has a relatively short half-life. The existence of a dual hypothalamo - pituitary control system for gonadotrophin secretion is discussed.

We investigated the distribution of LRH in various tissues of the rat (chapter 7). The highest concentrations were measured in the stalk median eminence. Low or undetectable levels of immunoassayable LRH were found in the posterior pituitary, pineal gland and cerebral cortex. A relationship between body weight and hypothalamic LRH content was established. Removal of gonadal steroid feed-back was found to cause a reduction in the hypothalamic LRH content, probably due to a lowering of the ratio of biosynthesis to release. Two techniques were studied for possible use in investigations of the regulation of LRH release from the hypothalamus: a static incubation method and a perifusion method. Basal release of LRH could be measured, but no significant responses to biogenic amines were noted. Causes of the latter negative findings are discussed.

By means of a system for continuous monitoring of LH and FSH in women we could establish the lowest effective dose of LRH causing a significant release of LH and FSH (chapter 8). Dose-related responses could be induced in the release patterns of both gonadotrophins. The use of the releasing hormone as a diagnostic tool was investigated by measuring the responses to LRH stimulation in women with a variety of endocrinological disorders (chapter 7). Over-responsiveness as well as insufficient responses were noted in conditions with elevated and decreased endogenous LRH activity, respectively. A nasal spray mode of administration for LRH was investigated. The effectiveness of the method was checked. While establishing dose-response curves for LRH in man, we were often unable to achieve linear responses. By means of sequential injections spaced at different time intervals, we could establish that this was an effect of pituitary refractoriness to releasing hormone stimulation. The implications of this effect are discussed.

## 10.2 Nederlands

Wij hebben een radioimmunologische bepalingsmethode voor LRH ontwikkeld, getest en toegepast. Hiervoor hebben we een methode gevonden voor de bereiding van geschikte antilichamen (hoofdstuk 3). Daartoe werd LRH gekoppeld aan runderserum albumine (BSA). Dit complex werd toegediend aan konijnen door middel van intradermale "multiple site" injectie. We kozen als tracer LRH gelabeled met radioactief jodium gelabeled LRH als tracer hetgeen bereid werd met de Chloramine T methode. Een hierop volgende zuiveringsstap met Sephadex gel-chromatographie leverde een geschikt tracer preparaat voor de radioimmunologische bepaling van LRH.

We hebben de optimale omstandigheden voor de uitvoering van de radioimmunologische bepaling van LRH bepaald, te weten incubatietijd, incubatietemperatuur en concentraties van antiserum en radioactief gelabeled LRH (hoofdstuk 4). Twee verschillende methoden voor de scheiding van gebonden en vrij antigeen werden onderzocht, de "double antibody" techniek en een antilichaam-precipitatie methode. De laatstgenoemde techniek werd verkozen voor routine gebruik. De bepaling van standaard curven wordt gedetailleerd besproken. Een fase scheidingsmethode werd gekozen, en het type incubatieschema met de beste resultaten werd verkozen Drie verschillende antisera werden vergeleken en de nauwkeurigheit in de bepalingsmethode werd vastgesteld.

De specificiteit van de radioimmunoassay werd onderzocht. De immunologische specificiteit werd vastgesteld door middel van meting van het vermogen van LRH-analogen om gelabeled LRH uit zijn binding met antilichamen te verdringen (hoofdstuk 5). Twee van de drie geteste antisera hadden een hogere graad van specificiteit dan de derde, doordat ze de beide uiteinden van het LRH-molekuul nodig hadden voor binding. Het derde bleek alleen specifiek te zijn voor het C-uiteinde van het molekuul. Ook kon worden vastgesteld, dat de hoge specificiteit voor langere tijd bleef bestaan gedurende de productie van antiserum. Om de biologische specificiteit te controleren werd een bioassay voor LRH ontwikkeld. Op zijn beurt maakte deze een radioimmunoassay van rat-LH nodig. De verkregen resultaten toonden uitstekende overeenstemming tussen de verschillende bepalingsmethoden. Meer gegevens bevestigden de validiteit van de radioimmunoassay. Zo werd in experimenten vastgesteld dat de inhibitiecurven van hypothalamus extracten en synthetisch LRH parallel verlopen, beschreven in hoofdstuk 7.

De weg van direkte meting van LRH in plasma door middel van radioimmunologische bepaling is bezaaid met voetangels en klemmen (hoofdstuk 6). Proteolytische enzymen in het plasma kunnen LRH afbreken en andere (makromoleculaire) plasma componenten kunnen de

bepaling storen en en onbetrouwbar maken. De a-specifieke storing kan grotendeels te niet gedaan worden door gebruikmaking van een door ons ontwikkelde en beproefde extractie-procedure. Continue bemonstering is noodzakelijk bij meting van LRH in de bloedsomloop, omdat LRH op een pulsvormige wijse wordt afgescheiden en het ook een betrekkelijk korte halfwaardetijd heeft. Het bestaan van een dubbel hypothalamo - hypophysair regelsysteem voor de secretie van gonadotrophinen wordt besproken.

We onderzochten de verspreiding van LRH in verschillende weefsels van de rat (hoofdstuk 7). De hoogste concentraties werden gevonden in de mediane eminens. Lage of onmeetbare concentraties werden gemeten in de hypofyse-achterkwab, de epifyse en de hersenschors. Een verband tussen lichaamsgewicht en LRH-gehalte van de hypothalamus werd vastgesteld. Het effekt van blokkering van de feed-back van de geslachtshormonen bleek een verlaging van de LRH tot gevolg te hebben. Waarschijnlijk werd dit veroorzaakt door een verlaging van de verhouding tussen biosynthese en secretie van het LRH. Twee technieken werden onderzocht voor gebruik bij het in onderzoek van de regulatie van de LRH secretie door de hypothalamus: een statisch incubatie-systeem en een perifusie-methode. Basale secretie van LRH kon gemeten worden, maar een significante respons op biogene aminen werd niet vastgesteld. Oorzaken van deze bevindingen zijn besproken.

Door middel van een systeem, waarbij LH en FSH bij vrouwen konstant werden gemeten, konden we de laagste effektieve dosis LRH vaststellen, die signifikante secretie van LH en FSH kan induceren (hoofdstuk 8). Een dosis-afhankelijke respons kan geinduceerd worden in de secretiepatronen van beide gonadotrophinen. Het gebruik van LRH als een diagnostisch middel werd onderzocht door middel van meting van de respons op LRH-stimulatie bij vrouwen met een verschillende endocrinologische afwijkingen. Overmatige respons en onvolledige respons werden opgemerkt in omstandigheden met verhoogde respectievelijk verlaagde endogene LRH activiteit. Een vorm van toedienen van LRH door middel van een neus-spray werd ook onderzocht. Tijdens de bepaling van dosis-respons curven voor LRH bij de mens vonden we, dat het vaak onmogelijk was om lineaire responsen te verkrijgen. Door sequentiaale injecties met bepaalde tijdsintervallen te geven, konden we vaststellen dat dit het gevolg is van een refractaire periode van de hypofyse als reaktie op LRH stimulatie. De implicaties van dit effekt worden besproken.

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## CURRICULUM VITAE

Hans G. Dahlen was born on 1.XII.1943 in Karlstad, Sweden. After primary schools he underwent training as laboratory technician in Skoghall and worked there briefly in organic synthesis. He then moved to Stockholm in 1961 and took a degree in chemical engineering at the Technical Institute of Stockholm in 1964. Military service in the Swedish Army was followed by a tour of duty in the United Nations Peacekeeping Forces in Cyprus. Immatriculation at the University of Strathclyde, Glasgow was next and led to earning the degree of Bachelor of Science (honours) in biochemistry in 1969. He was employed as chemotechniker by Boehringer Sohn in Ingelheim/Rhein, West Germany 1969 - 1971 and then moved to the University of Ulm in Ulm/Donau, West Germany, as head of the Peptide Hormone Laboratories until 1977. This was followed by employment as New Product Developing Chemist and Associate Research Manager by J.T.Baker Chemicals, Deventer until 1983. Two years later, in 1985, he received the degree of Master of Science in chemistry (doctoraal examen) from the University of Nijmegen.

# STELLINGEN

1) Although the classical translocation model for steroid - receptor interactions has been convincingly disproven, the concept of an exclusive nuclear localization of both occupied and un-occupied receptor needs further validation.

Raam et al. Eur.J.Cancer.Clin.Oncol. 18:1,1982
Raam et al. Breast Cancer Res.Treat. 3:179,1983
Welshons et al. Nature 307:747,1984
Welshons et al. Endocrinology 117:2140,1985
Roberstson et al. Endocr.Soc.Meeting 1985, Abstract Book, p20

2) The mode of administration will determine if LH-releasing agonists will act in a stimulatory or inhibitory manner.

Sopelak, Collins & Hodgen, J.Clin.Endocr.Metab. 65:557,1986
Crowley et al. Rec.Progr.Horm.Res. 41:473,1985
Monroe et al. Fertil.Steril. 43:361,1985

3) Hyperprolactinaemia need not result in galactorrhea and even in its mild or transient forms it may cause amenorrhea.

Bohnet et al. Clin.Endocrinology 5:25,1976
Ben-David et al. J.Clin.Endocr.Metab. 57:442,1983
Suginami et al. J.Clin.Endocr.Metab. 62:899,1986

4) The presence of oestradiol and oestradiol receptors in Saccharomyces cerevisae implies a physiological function of these compounds in yeast. However, since the presence of oestradiol may in fact be due to contaminated media components, caution should be exercised in interpreting the results.

Feldman et al. Science 224:1119,1984
Miller et al. Endocrinology 119:1362,1986

5) The statement that "in order to induce potassium sensitivity of the phosphorylated intermediate of Na/K ATPase, a simultaneous binding of sodium is required", is in error.

Siegel et al. J.Biol.Chem. 255:3935,1980

6) A bioassay for moult inhibiting hormone can be invalidated by the fact that a wound reaction might be as likely to induce apolysis as does ecdysone.

Freeman & Costlow, J.Exp.Zool. 210:333,1979
Willems & van Deijnen, personal communication

7) As the degree of acetylation is important for the biological activity of melanocyte stimulating hormone is it not sufficient to measure only total circulating levels of the peptide.

Kawauchi et al. Gen.Comp.Endocr. 53:37,1984
Pickering et al. Gen.Comp.Endocr. in press 1986

8) When many authors of scientific publications are saying "abbreviations" they are in fact frequently refering to what should be named acronyms.

9) One of the side effects of nuclear power is it's ability to induce muddled thinking.

Twello 27 October 1986 Hans G. Dahlen